Improvement of Thermal Stability of Freeze-Dried Protein Embedded in Sugar(Dissertation_全文)

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Contents

Chapter 1

Introduction

1. 1 Introductory remarks

When sugar solution is dehydrated, hydrogen bonds among sugar molecules are formed randomly. As a result, sugar molecules form amorphous structure easily. The amorphous structure formed by sugars is structurally flexible and thus can include other molecules. Hence, amorphous sugars are used as bulking agents and dilution for valuable ingredients. Besides the inclusion effect, amorphous sugars are known to be effective in protecting labile components against physical and chemical changes. Amorphous sugars are used also as stabilizers for labile components using those effects. Especially in the case that labile proteins are used for commercial products, they are often embedded in amorphous matrices of sugars.

Recently, by intensive and extensive researches of bioscience and biotechnology, numerous proteins that have possibilities to be excellent medical ingredients have been found. Concomitant with it, the products containing proteins in amorphous sugars are expected to increase. The importance of the prediction and improvement of the quality of the sugar-protein products will increase.

The quality of the sugar-protein products depends mainly on the two stabilities. One is the stability of protein during manufacturing process and storage, and another is the stability of amorphous structure formed by

sugars. The loss of bioactivities of proteins decreases the efficiency of the drugs. Collapse of amorphous structures of sugars also decreases the product quality seriously. The glass transition and the subsequent crystallization of amorphous matrix of sugar cause the loss of appearance, texture and stabilization of proteins.

As described above, in order to obtain sugar-protein products with high quality and long shelf-life, we must improve these two stabilities. The understanding of the mechanism of stabilization of protein by sugar is indispensable for improving the stability of proteins. To improve the stability of amorphous matrix of sugar, we need to understand influences of protein included on the stability of amorphous sugars.

1. 2 Literature review and problems

1. 2. 1 Degradation and stabilization of protein

Proteins are macromolecules composed with amino residues, and many of them have excellent bioactivities. The bioactivities mainly depend on higher order structures formed exquisitely by amino residues. Thus the change of the higher order structures of proteins causes the loss of bioactivities.

Proteins are often degraded by proteolysis, deamidation, oxidation, racemination, and the formation of incorrect disulfide bonds (Manning et al., 1989). It is known that these chemical degradation is reduced at low temperature and water activity. Proteins are often freeze-dried to prevent the chemical degradation. However, some proteins are unstable and their activities are lost during freeze-drying and storage. Denaturation, aggregation, precipitation, and adsorption to surfaces are known as the

physical degradation. It is reported that denaturation occurs first, and chemical degradation is induced by the denaturation (Manning et al., 1989; Stevenson et al., 1993).

During freeze-drying, the phase around proteins turns from liquid to frozen solid and reaches dry solid. In each state and each transformation, proteins suffer denaturation. Sugars are known to be effective in stabilizing proteins against denaturation at every case. The author reviewed researches on mechanism of denaturation of protein and stabilizing effects of sugars as follows.

1. 2. 1. 1 Denaturation and stabilization of proteins in solution

In aqueous solution, denaturation of protein is caused by both increase and decrease in temperature. Thermal motion of solvent molecules and amino residues of protein increase with temperature, resulting in a conformational change of protein (Gekko, 1985). Decrease of temperature induces an ordination of water interacting to protein. Consequently, the ordinations of the hydrated water molecules cause the conformational change of protein because most interactions supporting protein structure are formed through the hydrating water in aqueous solution (Brandts, 1968).

It is reported that many solutes such as polyols, amino acids, methylamines, and lyotropic salts as well as sugars are effective in minimizing the denaturation of proteins (Gekko and Morikawa, 1981; Gekko and Timasheff, 1981; Lee and Timasheff, 1981; Arakawa and Timasheff, 1982a, b, 1983, 1985). As for the mechanism of stabilizing proteins by those solutes, Timasheff and Arakawa (1982a, b) investigated the preferential interaction of proteins with solvent components using high-precision densimetry, and explained the stabilization of proteins thermodynamically. Solute molecules are preferentially excluded from the domain of the protein

and the protein is preferentially hydrated. By the preferential hydration, conformational change of protein becomes thermodynamically unfavorable.

1. 2. 1. 2 Denaturation and stabilization of proteins during freezing

The denaturation of protein during freezing is considered to be caused mainly by dehydration of proteins. When ice crystal grows to a hydration shell on protein, the hydrated water molecules should be arranged following the order of water molecules in ice. The ordination of hydrated water influences the interactions that support protein structure, resulting in conformational change of protein (Hanafusa, 1970; Sahara, 1989). Besides the change of hydration state of proteins, influence of salts concentrated by freezing, the change of pH, mechanical stress with growth of ice crystal, and formation of disulfide bond have been suggested (Chilson et al., 1964).

Denaturation of protein by freezing can be minimized by the same solutes those used in aqueous system (Crowe et al., 1990). The mechanism of protection of protein during freezing has been explained. Hanafusa et al. (1987) indicated that proteins directly interacted to solute molecules at freezing, and the direct interactions maintained the protein structure. On the other hand, Crowe et al. (1990) explained the stabilizing effect by the preferential hydration of protein as same as the stabilization in aqueous system. Now the latter explanation seems to be accepted in general.

1. 2. 1. 3 Denaturation and stabilization of protein during dehydration

During dehydration of frozen protein solution, denaturation of protein is caused by the removal of hydrated water that contribute to keep the protein structure.

Only certain solutes such as sugar, carbohydrate, and some amino acids, are known to be effective in minimizing the denaturation due to dehydration.

As for the mechanism of the stabilizing effect of sugars against dehydration stress, two hypotheses have been proposed. One is the water substitution hypothesis (Carpenter and Crowe, 1988, 1989) and another is glassy state theory (Franks et al., 1991; Levine and Slade, 1992; Franks, 1994). Water substitution hypothesis means that sugar molecules form hydrogen-bonds to the dried protein instead of water molecules to keep higher order structure of the protein, and so the "water substitution" may protect the protein from the drying stresses. On the other hand, glassy state theory suggests that the protein is effectively stabilized in an amorphous matrix. In the amorphous matrix, the viscosity is so high that physical and chemical degradation are essentially prevented. Although these two hypotheses have been verified well experimentally, the relation between two hypotheses has not been elucidated yet.

1. 2. 1. 4 Denaturation and stabilization during storage

During storage, freeze-dried proteins denature in two ways. One is that further removal of hydrated water during storage induces a conformational change of protein, and another is that excessive thermal vibration causes a change of protein structure.

The stabilizing effect of sugars against the dehydration stress during storage is also explained using the two hypotheses, water replacement hypothesis and glassy state theory (Franks et al., 1991; Izutsu et al., 1991). On the other hand, the stabilization of dried proteins by sugar against thermal denaturation has been rarely studied (Izutsu et al., 1991) although the thermal degradation of protein is important in many cases. Consequently, the mechanism of thermal stabilization of dried protein by sugar has not been elucidated yet.

1. 2. 2 Physical stability of amorphous structure of sugar

Amorphous sugars are stable under limited conditions. Amorphous structure is supported by its viscosity. When the viscosity decreases to too low to support the matrix, structural changes such as shrinkage and collapse occur (Bellows and King, 1972).

The viscosity of amorphous sugar decreases with increasing temperature. Amorphous solids turn into liquid-like rubber above a certain temperature, resulting in collapse. The temperature is called collapse temperature (T_C)

Collapse of amorphous sugar is also caused by the increase of humidity. Generally, amorphous sugar has high water sorption affinity, and thus moisture content of amorphous sugar increases remarkably in a humid atmosphere. Because water plasticizes amorphous sugar, the increase of moisture content lowers the viscosity and results in collapse.

As described above, insights for T_C and sorption behavior are indispensable for understanding the physical stability of amorphous structure of sugar. Numerous studies discussing these points have been reported. The author reviewed such researches as follows.

1. 2. 2. 1 Collapse and glass transition of amorphous sugar

Bellows and King (1972) determined T_C of several amorphous sugars by microscopic observations. They proposed that the collapse was caused by decreasing the viscosity of the amorphous matrix below 10^7-10^{10}cp. The dependency of T_C on moisture and solute contents was studied by Tsourouflis et al. (1976). Those results are useful to understand the collapse phenomena.

In polymer science, it is well known that an amorphous polymer solid turns into liquid-like "rubber", and that the viscosity decreases drastically above a certain temperature. This phenomenon is "glass transition," and the temperature is called "glass transition temperature (T_g)". To and Flink (1978) measured T_g of freeze-dried sugars by some thermal analyzing techniques and compared the T_g values to T_c obtained by visual observations. They found that T_g and T_c were in good agreement and concluded that the collapse is ascribed to the glass transition of amorphous sugar.

Since the study by To and Flink (1978), the physical stabilities of various amorphous sugars have been evaluated by T_g values. Those values are shown in Table 1. 1. Compared the values reported for the same sugars, there are significant differences among them. This is because the researchers used different methods to prepare samples and to determine T_g. Furthermore, T_g of whole kinds of sugars has not been measured. Consequently, there is serious lack of the knowledge on differences in physical stability of amorphous structure among sugars.

Substances coexisting in sugars such as proteins and water influence physical stability of amorphous sugar. The influence is important to use amorphous sugars for commercial products. Because the most popular coexistence in amorphous sugar is water, the dependency of T_g on moisture content has been investigated extensively (Slade and Levine, 1988; Green and Angell, 1989; Orford et al., 1990; Roos and Karel, 1990, 1991a,b,c,d). From these studies, it has been known that T_g values of amorphous sugars decrease with increasing moisture content.

Table 1.1 Glass transition temperatures (T_g) of various sugars

sugar	T_g [°C]
arabinose	4[a], -2[b]
xylose	13[a], 6[b], 10[c]
ribose	-11[a], -20[b]
sorbose	19[b]
galactose	32[a], 32[b], 30[g]
fructose	32[b], 26[e]
glucose	38[a], 31[b]
mannose	30[a], 25[b], 36[c]
ribose	-10[c]
rhaminose	23[a], -7[b]
lactose	101[d]
maltose	92[c], 87[e]
isomaltose	78[a]
sucrose	70[a], 62[b], 66[c], 57[f], 52[g]
turanose	52[c]
nystose	77[c]
cellobiose	77[c]
trehalose	79[c], 100[b], 77[g]
mannobiose	90[c]
melibiose	95[a]
maltotriose	134[a], 76[c]
maltotetraose	112[c]
maltopentaose	125[c]
maltohexaose	175[a], 134[c]
maltoheptose	139[c]

Values were reported by (a) Orford et al (1990), (b) Roos (1993), (c) Slade and Levine (1988), (d) Roos and Karel (1991b), (e) Roos and Karel (1991a), (f) Roos and Karel (1991c), and (g) Green and Angell (1989)

Slade and Levine (1988), and Orford et al. (1989) examined T_g of the mixture of different kinds of sugars. Roos and Karel (1991a) investigated the influence of the addition of carbohydrates on T_g. These researches indicate that, in most case, T_g increases with the component of higher molecular weight but decrease with the component of low molecular weight. te Booy et al. (1992) investigated the influence of various solutes such as amino acids, citric acids, alcohol, and salts, and reported that these solutes also influenced T_g values.

According to these reports, it is expected that the presence of proteins also influence T_g values. However, the influence of proteins on T_g values has never been studied.

1. 2. 2. 2 Sorption behavior of amorphous sugar

For certain sugars such as glucose, sucrose, maltose, and lactose, sorption behavior of their amorphous matrices have been investigated by measuring moisture contents at certain water activities (Makower and Dye, 1954; Roos and Karel, 1990, 1991a,b,c,d). However, sorption behavior of some sugars are obscure in spite of the industrial importance.

For the industrial application, the insight into the influence of coexistence on sorption behavior is also important. Lang and Steinberg (1980) investigated sorption isotherms of some solute-solute mixtures. On the basis of the results, they calculated the moisture contents of the mixture at a specified water activity from the moisture content of each component as Eq. 1.

$$w_{cal} = C_A \times w_A + C_B \times w_B \qquad (1)$$

where w_{cal} is the calculated moisture content of the mixture at a specific water activity, C_A and C_B are the weight ratio of component A and B in the

mixture, g-component/g-dry matter. w_A and w_B are moisture contents of component A and B alone at a specified water activity, g-water/g-dry matter.

Chinachoti and Steinberg measured the sorption isotherms of sucrose-starch (1984) and sucrose-NaCl mixtures (1985). They compared the experimental isotherms with those calculated from the Lang-Steinberg equation given by Eq. 1 and found that each solute caused decrease in moisture content. This is because the interaction between solutes affects the sorption behavior. Thus, for accurate correlation and prediction of the sorption behavior of the solutes mixture, it is necessary to understand the influence of solute-solute interaction on sorption behaviors.

Understanding of the influence of proteins on sorption behavior would be of great value in controlling protein functionalities. There have been some reports on sorption behavior of sugar-protein mixture. Chinachoti and Steinberg (1988) measured sorption isotherms of amorphous sucrose containing casein, egg albumin, and gluten over the water activity range 0.58-0.93. They found that sorption isotherm of sucrose-protein showed higher than those calculated from the Lang-Steinberg equation. Bakhit and Schmidt (1993) also investigated sorption behavior of sucrose-casein mixture at water activity range of 0.23-0.93, and confirmed that coexisting of sugar and protein influenced the sorption behavior.

As described above, sorption behavior of sugar-protein mixtures is comparatively well investigated. However, there are serious lack of insight on the following points. These studies were done for comparatively high water activity range, and sugars except sucrose have been rarely investigated. Furthermore, the influence of sugar-protein interaction on the sorption behavior has not been elucidated.

1. 3 Objectives of this work

The guideline for the design of sugar-protein products and manufacturing process becomes necessary increasingly with advances in biotechnology. For the establishment of such a guideline, it is indispensable to elucidate the unclear aspects, that is, the thermal stabilizing effect of sugar on dried protein, the influence of protein on glass transition of amorphous sugar, and sorption behaviors of amorphous sugars containing protein.

In Chapter 2, the relation between the thermal stabilizing effect of sugar on freeze-dried protein and amorphous structure of sugars is investigated. Freeze-dried sugar-protein samples with various sugars and different degrees of crystallinity of the sugar are prepared. The relation between residual activities of proteins during storage and the degree of crystallinity of sugars is examined. Furthermore, by using same kind of sugar, the author prepares sugar-protein samples with different crystallinity of sugar, and investigate difference of stabilizing effect for them.

The relation between the thermal stabilizing effect and sugar-protein hydrogen bond is focused in Chapter 3. The degree of sugar-protein hydrogen bond formation and thermal stability of freeze-dried protein are investigated for samples with crystalline and amorphous sugar. The dependency of thermal stability of protein on sugar content is also investigated. On the basis of the insights obtained, a model of the thermal stabilization of proteins in amorphous sugar is proposed.

In Chapter 4, the influences of protein on sorption and glass transition behavior are investigated. Additionally, the author investigates the influence on other physical natures, crystallization temperature, melting temperature, and the enthalpies due to the phase transition.

In Chapter 5, the author investigated the influence of protein on glass transition behavior of sugar-protein mixtures for four kinds of sugars.

Finally, in Chapter 6, the author discusses the whole thesis to give conclusions. The perspectives in these subjects are also discussed.

1. 4 Publications on this thesis

Suzuki, T., K. Imamura, K. Yamamoto and M. Okazaki, Drying '96 - Proceedings of the 10th International Drying Symposium, vol. B, 1261-1266 (1996) (Chapter 2)

Suzuki, T., K. Imamura, K. Yamamoto, T. Satoh and M. Okazaki, Journal of Chemical Engineering of Japan, vol. 30, 609-613 (1997) (Chapter 2)

Suzuki, T., K. Imamura, H. Fujimoto and M. Okazaki, Journal of Chemical Engineering of Japan, vol. 31, 565-571 (1998) (Chapter 3)

Suzuki, T., K. Imamura, H. Fujimoto and M. Okazaki, Drying '98 - Proceedings of the 11th International Drying Symposium, vol. C, 1741-1747 (1998) (Chapter 3)

Imamura, K., T. Suzuki, S. Kirii, T. Tatsumichi and M. Okazaki, Journal of Chemical Engineering of Japan, vol. 31, 325-329 (1998) (Chapter 4)

Imamura, K., T. Suzuki, T. Tatsumichi, S. Kirii and M. Okazaki, to be submitted. (Chapter 5)

Although the two hypotheses are different in describing the stabilizing mechanism, the author thinks that these two hypotheses are complementary for description of phenomenon of stabilization effect of sugars. Hydrogen bond between sugars and proteins should be essential for stabilization effect of sugars. Hence it is quite natural that one expects water substitution occurs in freeze-dried samples. On the other hand, such water substitution will occur more easily when the degree of crystallinity of sugars is low than when the degree of crystallinity is high.

According to the consideration described above, there would be the tendency that freeze-dried proteins are stabilized with amorphous matrix formed by sugars. Hence the author is interested in the relation between the degree of crystallinity of sugars and stabilizing effect. Similar idea, that amorphous matrix of sugars stabilizes freeze-dried proteins, is also argued by glassy state theory. However, little is known about the relation between crystallinity of sugars and stabilizing effect of them. For example, to what degree does the stability of freeze-dried samples coincide with the order of the degree of crystallinity of sugars? Moreover, it is not known whether the stabilizing effect of a certain sugar changes by changing the degree of crystallinity of the sugar in the freeze-dried sample. Our present study is a preliminary one to discuss such problems.

The author studies the high-temperature inactivation of proteins freeze-dried with various sugars. Alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH), and malate dehydrogenase (MDH) were used as model proteins. Enzymes were freeze-dried with sugars and stored in dry air at 65°C. The thermal stabilities of enzymes were evaluated with the change in the residual activities during storage. The degree of crystallinity of sugars in freeze-dried samples was determined with X-ray powder diffractometry. Relationship between residual activities of enzymes during storage and the degree of crystallinity of sugars was examined. Furthermore, by using

sucrose the author investigates difference of stabilizing effect for samples of same sugar but different degree of crystallinity.

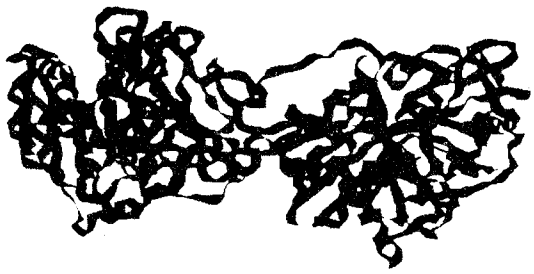
2. 2 Experimental

2. 2. 1 Materials

ADH was purchased from Sigma Chemicals Co. (St. Louis, U.S.A.). LDH and MDH were from Toyobo Co. (Osaka). Structures and characteristics of these enzymes are shown in Fig. 2.1. These enzymes were lyophilized powder and were used without further purification. All sugars used in this study, that is, glucose, sucrose, trehalose, maltose, lactose, raffinose, and maltotriose (Fig. 2.2) were obtained from Nacalai Tesque Inc. (Kyoto). Silica gel for desiccation, sodium pyruvate and ethanol for assay were from Wako Pure Chemical Industries Ltd. (Osaka). Glutathione was from Sigma and all other reagents were from Nacalai Tesque.

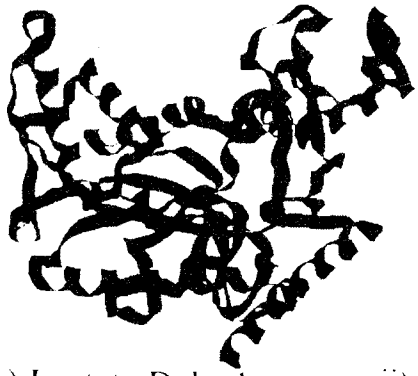
2. 2. 2 Freeze-drying and storage of enzymes

For each enzyme and each sugar, an aqueous solution containing 0.40 mg/ml enzyme and 0.055 mmol/ml sugar was prepared. Each 0.5 ml of prepared solutions was transferred to a glass test tube (inside diameter 6 mm, thickness 1 mm) and frozen in liquid nitrogen. The frozen samples were soaked in the ethanol bath of -10°C, and freeze-dried for 24 h at about 1 Pa. The sample containing only 0.40 mg/ml enzyme without sugars was also prepared in the same way.



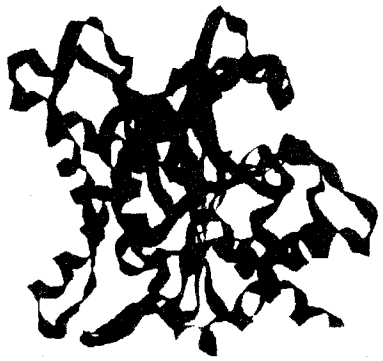
(a) Alcohol Dehydrogenase[i] (monomer)

Alcohol Dehydrogenase	
Enzyme code number	1. 1. 1. 1
Sourse	Horse Liver
Molecular Weight	approx. 80000
Structure	2 subunits
Number of amino residues	374 (monomer)



(b) Lactate Dehydrogenase[ii] (monomer)

Lactate Dehydrogenase	
Enzyme code number	1. 1. 1. 27
Sourse	Microorganism
Molecular Weight	approx. 140000
Structure	4 subunits
Number of amino residues	333 (monomer)

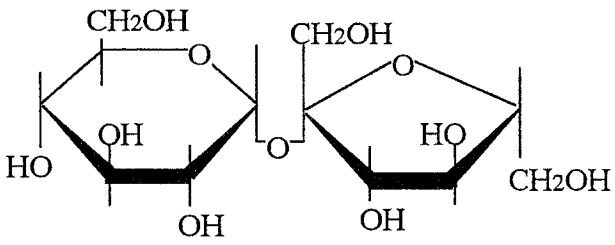


(c) Malate Dehydrogenase [iii] (monomer)

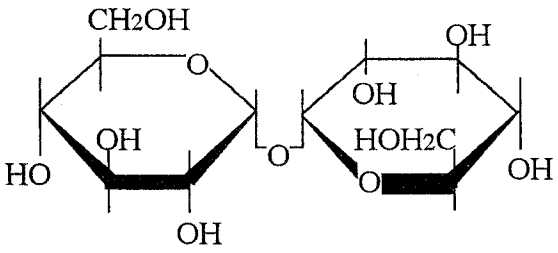
Malate Dehydrogenase	
Enzyme code number	1. 1. 1. 37
Sourse	Pig Heart
Molecular Weight	approx. 70000
Structure	2 subunits
Number of amino residues	312 (monomer)

Fig. 2.1 Enzymes

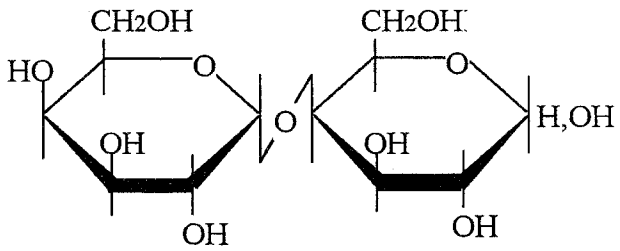
i) Eklund et al. (1981), ii) Ostendorp et al. (1996), iii) Hall et al. (1992)



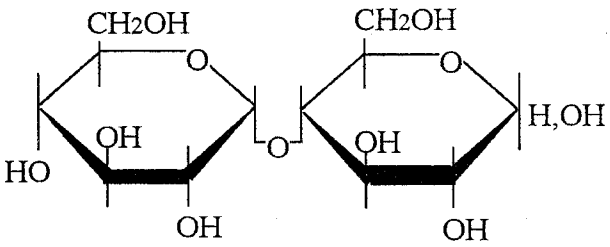
Sucrose



Trehalose

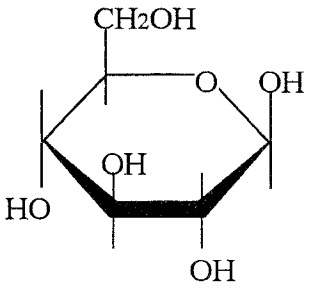


Lactose

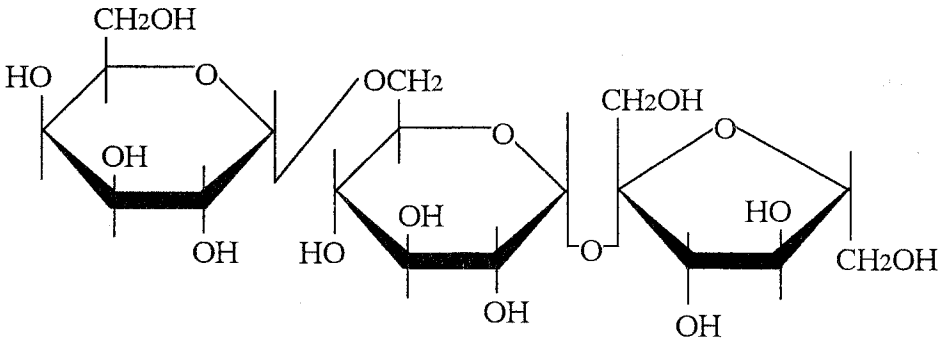


Maltose

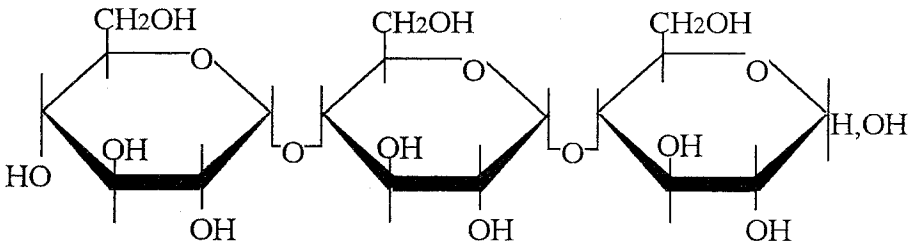
Fig. 2.2(a) Sugars (disaccharides)



Glucose



Raffinose



Maltotriose

Fig. 2.2(b) Sugars (mono- and trisaccharides)

Sucrose samples obtained by the method described above were high degree of crystallinity, that is, sucrose in samples was almost fully crystalline. In order to study the difference of stabilizing effect of sucrose among samples different in crystallinity, the author needed sucrose samples which were low degree of crystallinity. Hence such samples were prepared by the method as follows: 0.250 ml enzyme-sucrose solution (containing 0.40 mg/ml enzyme and 0.055 mmol/ml sucrose) was frozen in liquid nitrogen and was soaked in -30°C ethanol bath and freeze-dried for 48 h at 1 Pa. Then the sample was freeze-dried soaking in -10°C ethanol bath for 24h.

After freeze-drying, moisture content was 5.4±0.3 wt% (wet basis) for all samples with sugars except samples with amorphous sucrose, and 6.9 wt% for samples with amorphous sucrose. Moisture contents of samples without sugars were 6.3 wt% for ADH, 8.5 wt% for LDH, and 6.9 wt% for MDH, respectively. These samples were stored in a desiccator at 65°C. Silica gel was used for desiccation of air in the desiccator. The changes of moisture contents of the samples containing ADH in storage period have been examined. Moisture contents decreased to less than 0.5 wt% in a few days. Moisture contents of the LDH or MDH samples should also decrease in storage periods, though it was not examined. After appropriate storage periods, enzyme activities were measured by the methods described in the next section.

2. 2. 3 Assays of enzyme activity

ADH was assayed at 25°C by the method as follows: ADH preparation was made by rehydrating freeze-dried ADH samples with distilled water to give ADH concentration of 0.40 mg/ml. The 20 μl of ADH preparation was added to the 3.0 ml reaction mixture containing 0.1 M sodium pyrophosphate buffer (pH 9.0), 76.5 mM semicarbazide, 0.58 M ethanol, 2.1 mM NAD^+ and 1.0 mM glutathione. After the addition of ADH

preparation, the increase in absorbance at 340 nm was monitored immediately by a Shimadzu UV2200 spectrometer. From the results, the initial reaction rate was determined, and ADH activity was estimated.

LDH activity was also measured at 25°C. 3.0 ml of the reaction mixture contained 67 mM potassium phosphate buffer (pH 7.4), 0.49 mM pyruvate and 0.1 mM NADH. The reaction was initiated by addition of 10 μl of LDH preparation prediluted to 1.33 μg/ml and monitored by measuring the decrease in absorbance at 340 nm.

MDH was assayed at 37°C. 3.0 ml of the reaction mixture contained 97 mM potassium phosphate buffer (pH 7.5), 0.49 mM oxaloacetate and 0.2 mM NADH. The reaction was initiated by addition of 10 μl of MDH preparation prediluted to 1.33 μg/ml and monitored by measuring the decrease in absorbance at 340 nm.

2. 2. 4 X-ray diffraction

X-ray diffraction measurements were carried out on a Rigaku Ultima$^+$ system with graphite monochromater and Cu-Kα radiation. The degree of crystallinity was determined by the Ruland method (Ruland, 1961).

2. 3 Results and Discussion

2. 3. 1 Activities of enzymes freeze-dried with sugars

In this study, enzymes in all samples did not lose activities during freeze-drying. The activities of freeze-dried enzymes during storage are shown in Fig. 2.3. The values of the ordinate indicate residual activities of enzymes, that is, relative values to the initial activity before freezing.

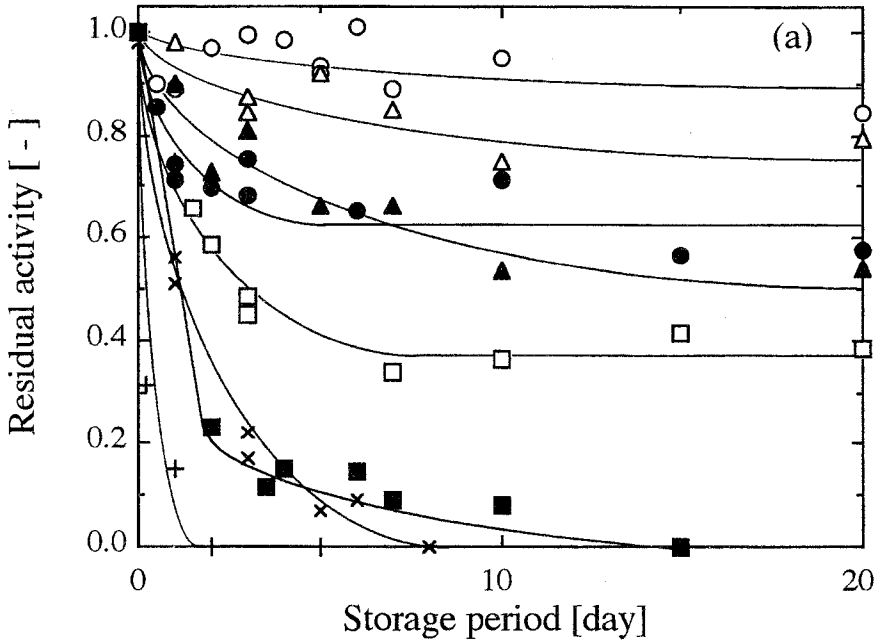


Fig. 2.3(a) Residual activity of ADH freeze-dried with (+) glucose, (□) sucrose, (○) trehalose, (●) maltose, (■) lactose, (△) raffinose, (▲) maltotriose, and (×) without sugars

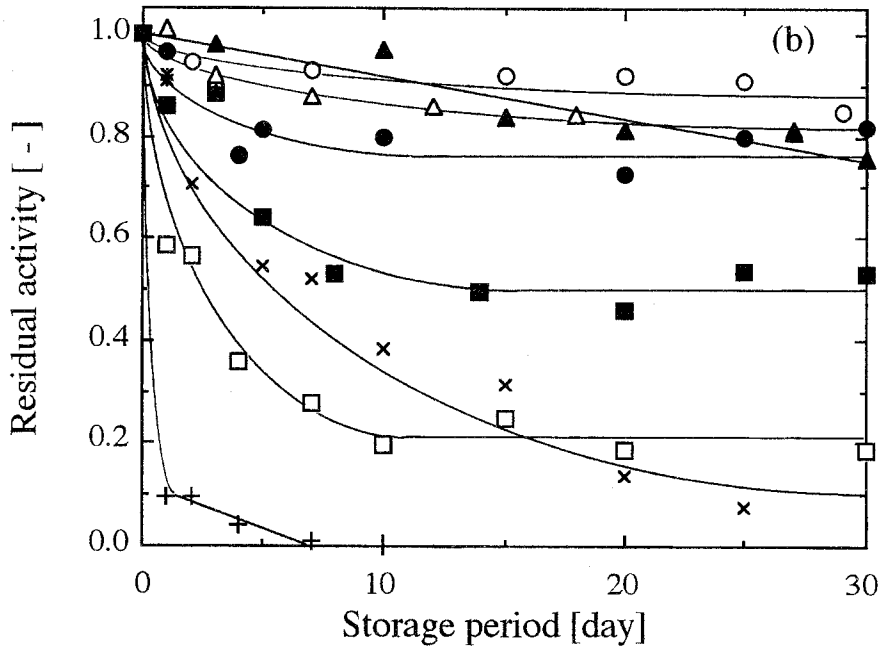


Fig. 2.3(b) Residual activity of LDH freeze-dried with (+) glucose, (□) sucrose, (○) trehalose, (●) maltose, (■) lactose, (△) raffinose, (▲) maltotriose, and (×) without sugars

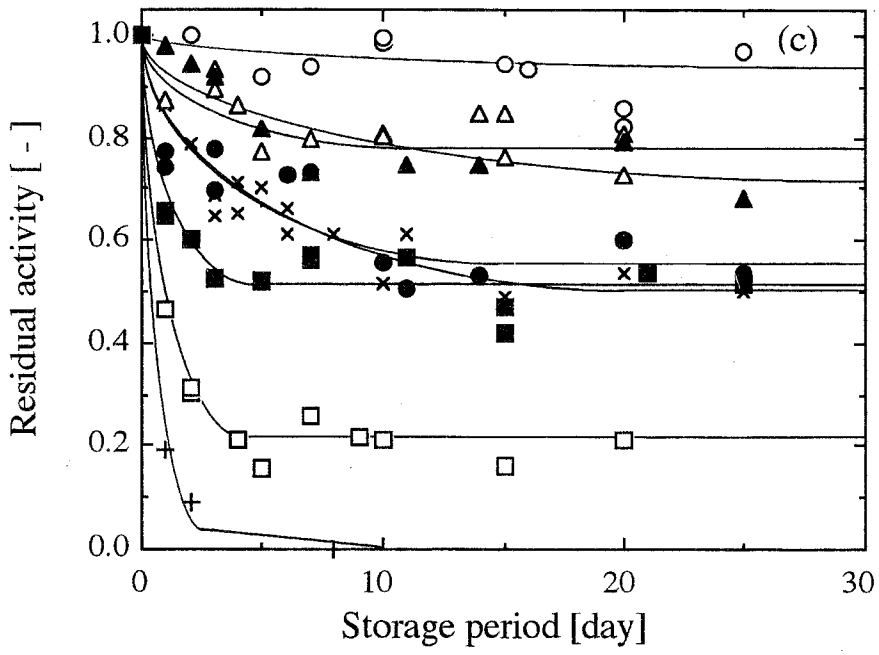


Fig. 2.3(c) Residual activity of MDH freeze-dried with (+) glucose, (□) sucrose, (○) trehalose, (●) maltose, (■) lactose, (△) raffinose, (▲) maltotriose, and (×) without sugars

From Fig. 2.3 we can see that activities of freeze-dried enzymes strongly depend on the kind of sugars added. Trehalose and raffinose were superior to keep enzyme activity. Addition of maltose and maltotriose were effective to stabilize ADH and LDH. Addition of lactose was effective only to LDH. Addition of sucrose was effective to ADH but inactivated MDH. Glucose inactivated three enzymes strongly. Figure 2.2 also shows remarkable inactivation of enzymes occurred for samples containing glucose, lactose, and maltose at early stages of storage periods. Actually, brown coloration was found for these samples. It is known that proteins with reducing sugars undergo the Maillard reaction which caused brown coloration at high temperature (Kato et al., 1986). Thus, it seems that not only the inactivation caused by deformation of the higher order structure but also the inactivation by the Maillard reaction occurred for the samples containing those sugars.

Originally, it was thought to be difficult to select additives for stabilizing dried proteins. Only a few kinds of solutes, such as sugars and amino acids, are effective for preserving proteins from inactivation caused by drying and storage at high temperature (Carpenter et al., 1987; Carpenter and Crowe, 1988; Crowe et al., 1990), as well as minimizing protein denaturation in aqueous systems and during freeze-thawing (Gekko and Morikawa, 1981; Gekko and Timasheff, 1981; Lee and Timasheff, 1981; Arakawa and Timasheff, 1982a, b, 1983, 1985). Moreover, according to the present results, only certain sugars can be used for stabilizing proteins. This confirms that selection of a proper sugar is both difficult and important.

2. 3. 2 Results of the measurement of X-ray diffraction

The X-ray diffraction patterns of freeze-dried samples are shown in Fig. 2.4. The values of degree of crystallinity determined from the X-ray diffraction

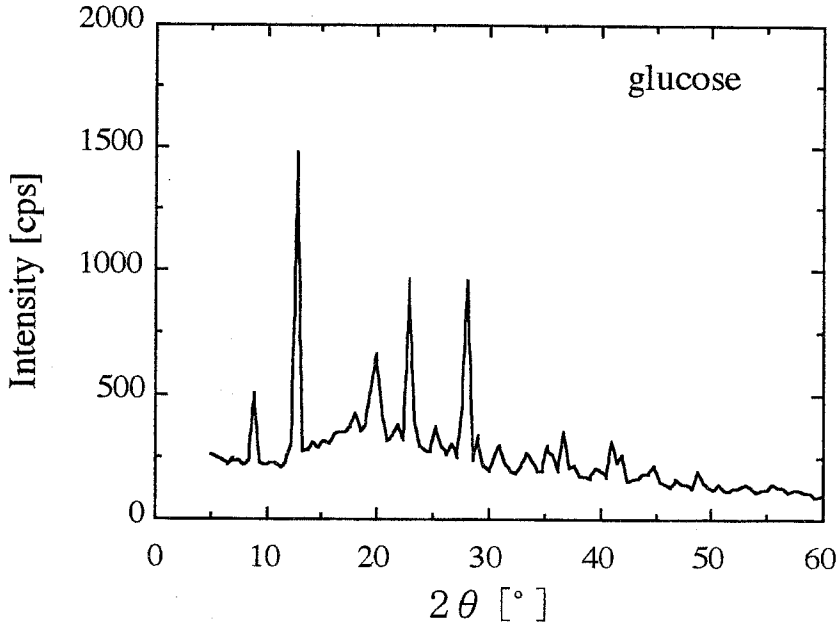


Fig. 2.4(a) X-ray powder diffraction patterns of freeze-dried ADH with glucose immediately after freeze-drying

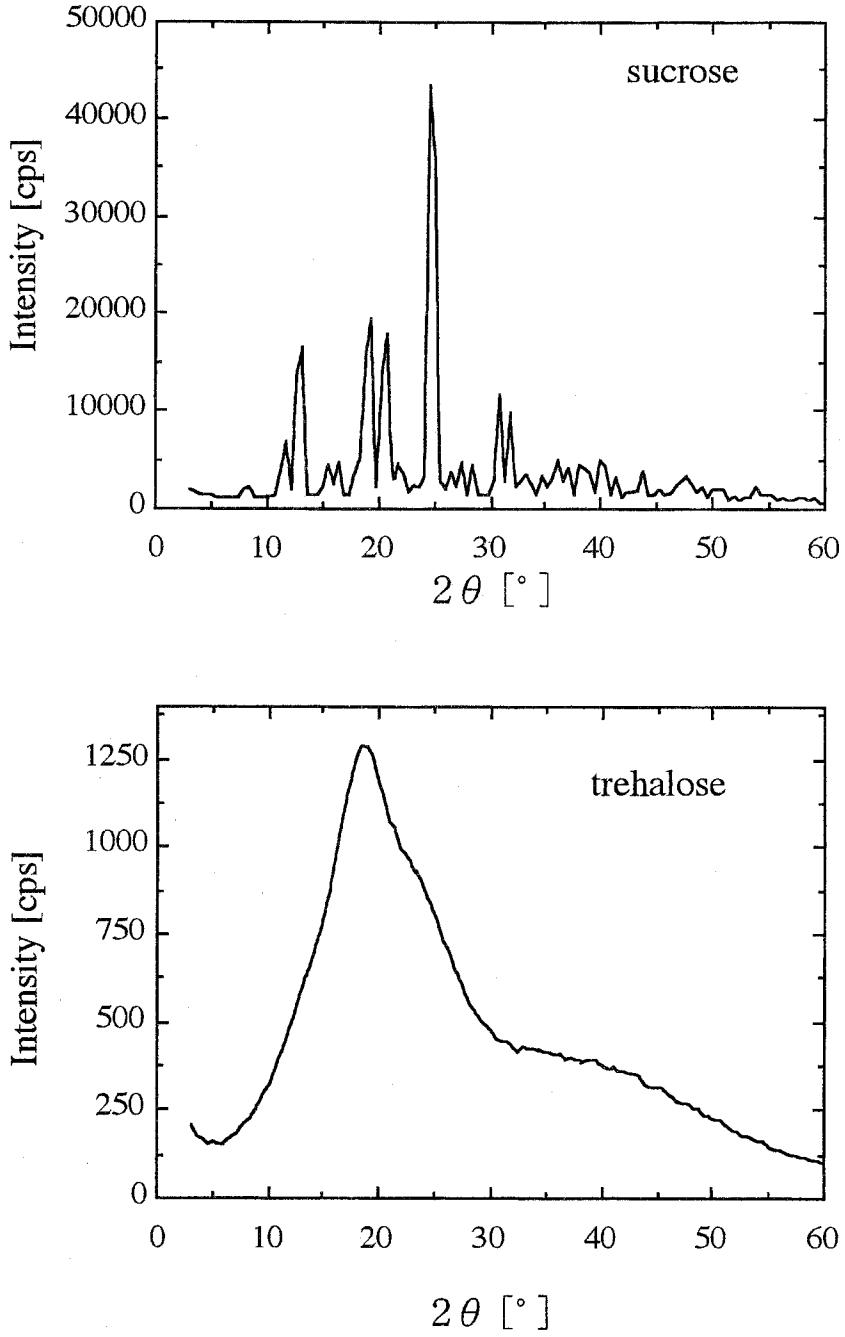


Fig. 2.4 (b) X-ray powder diffraction patterns of freeze-dried ADH with sucrose and trehalose immediately after freeze-drying

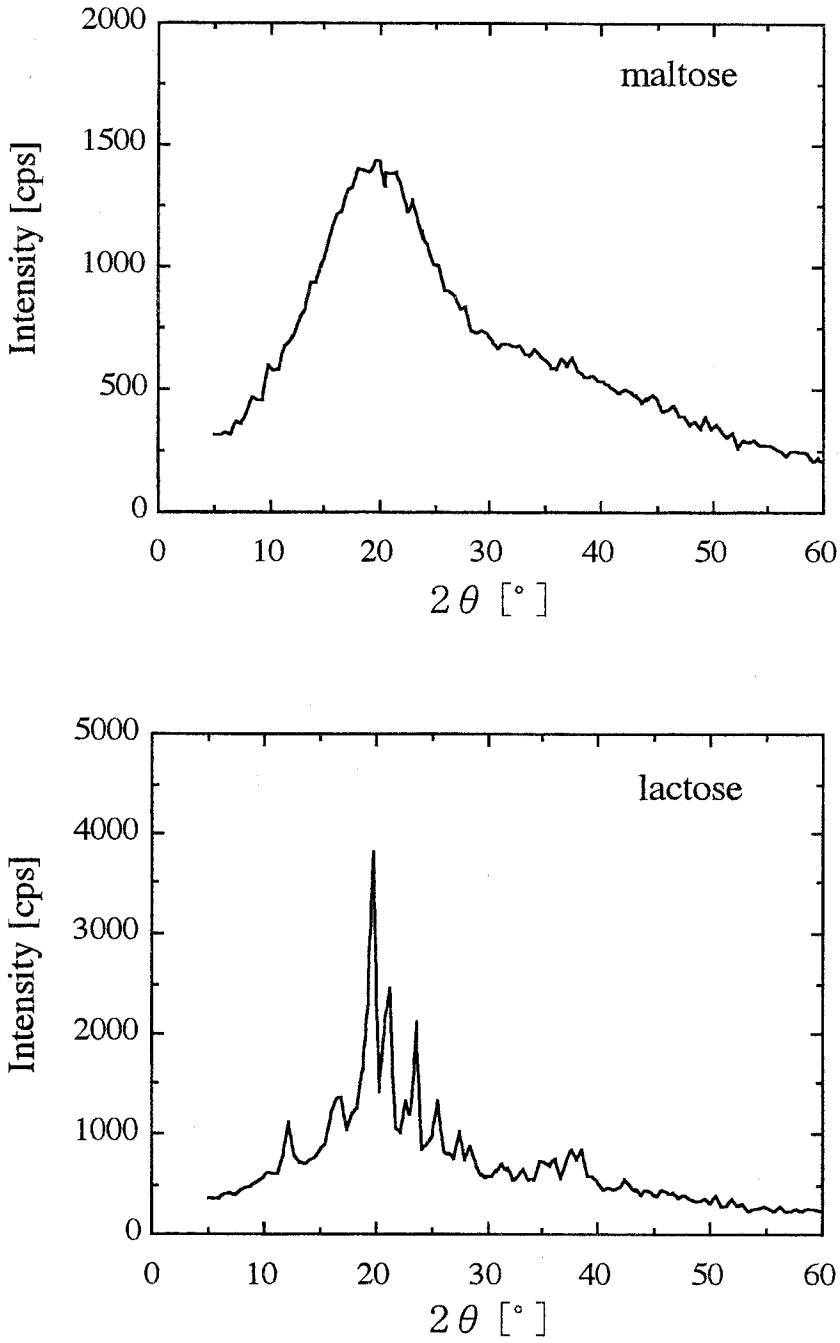


Fig. 2.4 (c) X-ray powder diffraction patterns of freeze-dried ADH with maltose and lactose immediately after freeze-drying

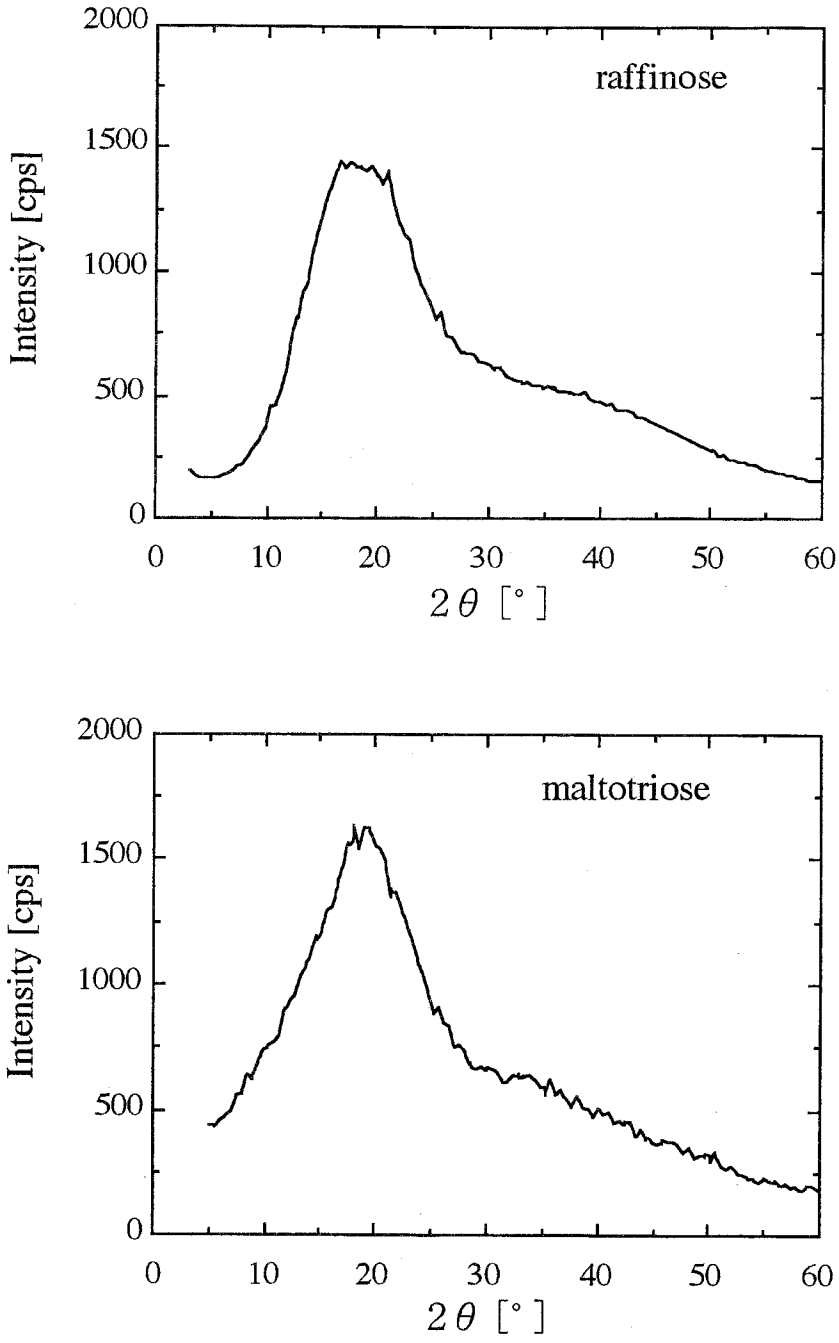


Fig. 2.4 (d) X-ray powder diffraction patterns of freeze-dried ADH with raffinose and maltotriose immediately after freeze-drying

patterns are summarized in Table 2. 1. The crystallinities of all freeze-dried samples did not change during storage.

According to the results, and taking account of the results of above section, it is found that there is a tendency that enzyme activity is preserved more in samples of low crystallinity than in samples of high crystallinity. Addition of trehalose and raffinose is remarkably effective to preserve the enzyme activity. As described above, the freeze-dried samples for these sugars are fully amorphous, judging from the results of measurement of X-ray powder diffractometry. Samples for maltose and maltotriose are also fully amorphous, and these sugars show stabilizing effects. In contrast, sucrose, lactose and glucose are inferior in stabilizing freeze-dried enzymes, and are found to be completely or partially crystallized in the freeze-dried samples.

Table 2.1 Degree of the crystallinity of freeze-dried ADH with sugars

sugar	degree of crystallinity (%)	
	after freeze-drying	during storage
glucose	49	-
sucrose	92	100 (15days)
trehalose	0	0 (15days)
maltose	0	0 (7days)
lactose	51	62 (7days)
raffinose	0	0 (15days)
maltotriose	0	0 (7days)

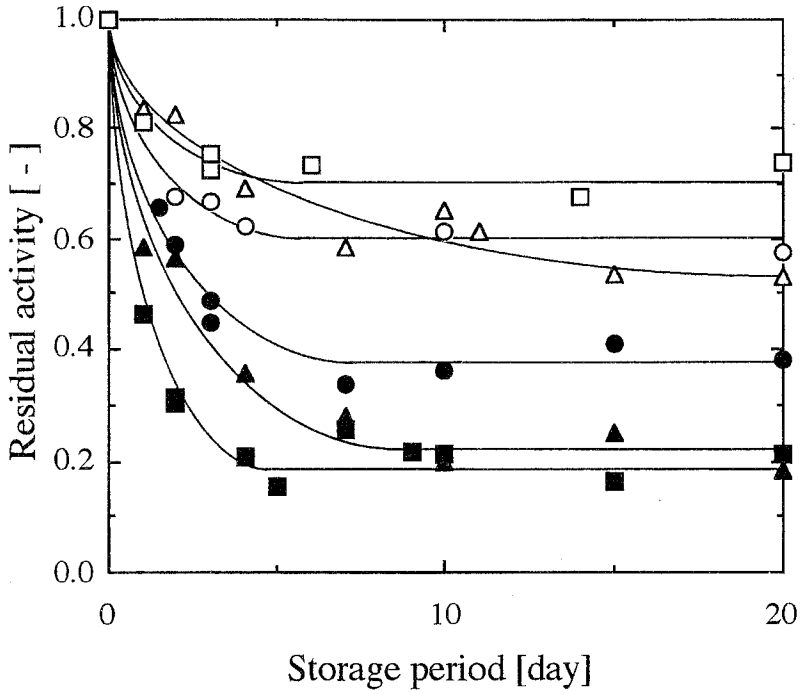


Fig. 2.5 Residual activity of (○ and ●) ADH, (△ and ▲) LDH, and (□ and ■) MDH in the samples of (open key) amorphous sucrose and (closed key) crystallized sucrose

2. 3. 3 Thermal stability of enzymes with amorphous sucrose

The results described above indicate that there should be close relationship between the stabilizing effect of sugars and the amorphous matrix of sugars. However, we can interpret the results in two ways: One way is that amorphous matrix formed by sugars should stabilize dried proteins. Another way is that sugars with low crystal-forming nature may stabilize dried proteins effectively, whether the sugars form amorphous matrix or crystallized structure. In order to clarify which interpretation is true, the author prepared freeze-dried enzymes with amorphous sucrose and evaluated thermal stability of enzymes during high-temperature storage.

In Fig. 2.5, the activities during storage of enzymes with amorphous sucrose are shown. Compared to samples of enzymes with crystal sucrose, the samples of enzymes with amorphous sucrose show remarkable high stability. This result confirms that the stabilization of dried proteins is caused by amorphous matrix formed by sugars.

As the author described in the introduction, the mechanism of stabilizing dried proteins by sugars is as follows. Proteins are surrounded by sugar molecules in freeze-dried samples. At the interface between protein and sugar molecules, the shape of interface formed by sugar molecules is a more suitable form in amorphous samples than in high crystallinity samples. Thus protein activity should be preserved more in amorphous matrix of sugars.

2. 4 Conclusion

The author investigated the relation between stabilizing effect of sugars on freeze-dried enzymes and the degree of crystallinity of sugars in the freeze-dried samples. It was found that sugars of low degree of crystallinity

preserved enzyme activities more than sugars of high degree of crystallinity. Furthermore, sucrose was found to stabilize enzymes effectively when sucrose formed amorphous matrix, though it showed low stabilizing effect when it was crystalline. These results indicate that the effect of stabilizing dried protein is caused by the amorphous matrix formed by sugars.

Chapter 3

Relation between Thermal Stabilizing Effect of Sugar on Protein and Sugar-Protein Hydrogen Bond

3. 1 Introduction

In the pharmaceutical industry, various proteins having excellent bioactivities are used as pharmaceutical raw materials. Such proteins are often manufactured in freeze-dried forms that are suitable for administration and storage. However, some proteins are unstable and lose their activity during freeze-drying and storage (Manning et al., 1989). Hence it is important to maintain activity of proteins during freeze-drying and storage. In order to achieve the purpose, sugars are often used for freeze-drying of proteins. Sugars are known to be effective in minimizing the dehydration stress in freeze-drying, and in stabilizing proteins during storage (Carpenter et al., 1987; Hanafusa, 1987; Levine & Slade, 1992).

In the previous chapter the author has studied the relation between the thermal stabilizing effect of sugars on freeze-dried enzymes and degree of crystallinity of the sugars. He has found that there is a tendency that sugars such as trehalose, maltotriose, etc. which show prominent stabilizing effects are amorphous in freeze-dried samples. Moreover, he has compared the stabilizing effect of amorphous sucrose with that of crystalline sucrose, and found that amorphous sucrose shows a remarkable stabilizing effect. From the results, it was clarified that amorphous matrix of sugar itself should contribute to the thermal stabilizing effect of sugar.

Now the author is interested in how freeze-dried protein is stabilized in an amorphous matrix of sugar. It is expected that the stabilizing effect of sugar is related to hydrogen-bonding between sugars and proteins. Though studies discussing such themes have been reported (Carpenter and Crowe, 1988 and 1989), the study discussing the relation between the thermal stabilizing effect of sugar and the hydrogen-bonding between sugar and protein have not been reported. Thus the author discusses this point in this chapter.

In this chapter, sucrose and lactate dehydrogenase (LDH) were used as a model sugar and a model protein. Freeze-dried samples of LDH involved in sucrose were prepared; they had different degrees of crystallinity of sucrose. The thermal stabilizing effect of sucrose was evaluated with the residual activity of LDH during high temperature storage. The degree of crystallinity of sucrose, glass transition temperature (T_g), and moisture content for samples were measured by X-ray diffractometry (XRD), differential scanning calorimetry (DSC), and Karl-Fischer moisture titration, respectively. Fourier transformation infrared (FT-IR) spectroscopy was used to evaluate the degree of formation of sucrose-LDH hydrogen bond. From these results, he discusses the contribution of the sucrose-LDH hydrogen bond to the thermal stabilizing effect of amorphous sucrose.

3. 2 Experimental

3. 2. 1 Materials

Sucrose was obtained from Nacalai Tesque, Inc. (Kyoto). LDH (Fig. 3.1) in the form of ammonium sulfate suspension was purchased from Toyobo Co. (Osaka). Sodium pyruvate was from Bio-Whittaker, Inc. (U.S.A.), and

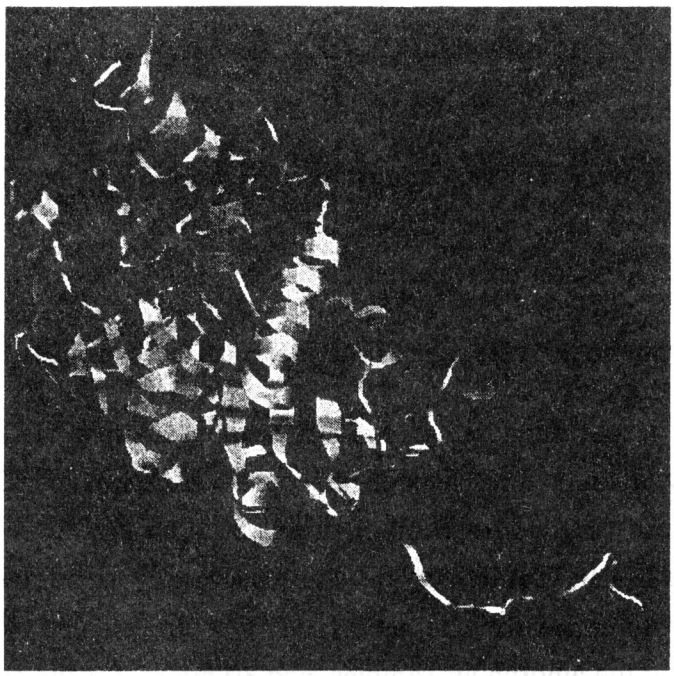
deuterium oxide was from Euriso-Top, CEA. (France). All other reagents were obtained from Nacalai Tesque, Inc.

LDH suspensions were dialyzed against 10 mM potassium phosphate, pH 7.5, for 6 h at 5 °C to remove ammonium sulfate before use. All other reagents were used without purification.

3. 2. 2 Freeze-drying and storage of the freeze-dried samples

Three kinds of freeze-dried samples, LDH without sucrose (LDH alone sample), LDH with amorphous sucrose (amorphous sample), and LDH with crystalline sucrose (crystalline sample), were prepared as follows. First, several sucrose-LDH solutions at different sucrose concentration were prepared. Sucrose concentration was 0 for LDH alone sample, 4, 10, 20, and 30 mg/ml for the amorphous samples, and 30 mg/ml for the crystalline sample. LDH concentration was adjusted to 3.5 mg/ml for all samples by measuring absorbance at 280 nm. According to our preliminary measurement, the extinction coefficient was 0.94 l cm^{-1} g^{-1}. 2.5 ml of each prepared solution was poured into a flask and frozen instantaneously with liquid nitrogen. Then the flask with frozen sample was soaked in an ethanol bath and freeze-dried at 1 Pa by the two drying steps in order to keep the sample temperature below T_g; samples were freeze-dried at -30°C for 24 h at the first step and at 0 °C for 9 h at the second step. After each of the two drying steps, residual moisture content of samples with 30 mg/ml sucrose was 0.052 and 0.016 g/g-sucrose.

After freeze-drying, the LDH alone sample and the amorphous samples were rehumidified over saturated CH_3COOK solution (RH 23%) in a vacuum desiccator at 25°C for 5 days. For preparation of the crystalline sample, a freeze-dried sample with 30 mg/ml sucrose was rehumidified at 25°C in a vacuum desiccator over saturated NaCl solution (RH 75%) for 4 h.



Lactate Dehydrogenase	
Enzyme code number	1. 1. 1. 27
Sourse	Pig Heart
Molecular Weight	approx. 115000
Structure	4 subunits
Number of amino residues	331 (monomer)

Fig. 3.1 Lactate Dehydrogenase[i)]

i) Grau et al., (1981)

Such humidification allowed full crystallization of sucrose in the sample. Then, water activity of the sample was adjusted by equilibrating over a saturated CH_3COOK solution. In order to prevent inactivation due to desiccation and oxidation during storage, approximately 2 mg portions of the obtained samples were sealed hermetically into DSC pans (aluminum, 20 μl) in nitrogen atmosphere and then stored at 65°C.

For evaluation of the formation of sucrose-LDH hydrogen bonds by FT-IR measurement, many unassigned bands due to water need to be eliminated from IR spectra. Hence the samples used for the IR measurement were prepared with deuterium oxide instead of distilled water using the method described above.

3. 2. 3 Assays of LDH activity

After an appropriate storage period, the LDH in the samples was assayed at 25°C by the following method. LDH preparation was made by rehydrating freeze-dried samples with 10 mM potassium phosphate (pH 7.5) to give an LDH concentration of 0.26 mg/ml. 10 μl of LDH preparation was added to the reaction mixture containing 67 mM potassium-phosphate buffer (pH 7.4), 0.49 mM pyruvate, and 0.1 mM NADH. After addition of the LDH preparation, the increase in absorbance at 340 nm was measured immediately by ultraviolet (UV) spectrometer (Shimadzu Co., UV2200). From the results, the initial reaction rate was determined, and LDH activity was estimated.

3. 2. 4 Analyses of the structure formed by sucrose

In order to evaluate the degree of crystallinity of sucrose in the sample before and during storage, XRD measurements were carried out using an Ultima$^+$ system (Rigaku Co.) with a graphite monochromater and Cu-Kα

radiation. DSC measurement was carried out to evaluate the physical stability of amorphous sucrose in the samples. A DSC (Rigaku Co., TAS-200) equipped with a cooling unit was used for the measurement of T_g.

3. 2. 5 FT-IR spectroscopy

Infrared (IR) spectra of samples were obtained in most cases as follows. Approximately 2 mg of the sample was mixed with 300 mg of dry KBr, and ground to a fine powder. The resulting powder was placed in a Perkin Elmer casting die (inside diameter 3 mm) and pressed into a pellet. The IR spectra of the pellets were taken with an FT-IR spectrometer (Perkin Elmer Co., Model 2000).

As described later, some of them showed crystallization of sucrose after 5 days storage. Due to crystallization of sucrose, water involved in amorphous sucrose was released from the crystallized region, and influenced the degree of formation of hydrogen bond. Hence, in order to examine the influence of the released water, the author also analyzed the sample re-equilibrated over saturated CH_3COOK solution at 25°C in a vacuum desiccator for 5 days prior to the measurement.

To obtain the IR spectra of fully hydrated LDH, a 7.0 mg/ml LDH solution was prepared using deuterium oxide and scanned by the ATR method using a horizontal ATR through plate system (Perkin Elmer Co.).

3. 2. 6 Moisture content analysis

Moisture contents of samples equilibrated over saturated CH_3COOK solution at 25°C in a vacuum desiccator were determined by using a Karl-Fischer titrator (Kyoto Electronic Manufacturing Co., MK210).

3. 3 Results and Discussions

3. 3. 1 Influence of sucrose content on stabilization of LDH

In the following sections the author distinguishes amorphous samples by their LDH-based sucrose contents, g-sucrose/g-LDH. Thus, the amorphous samples whose sucrose concentrations are 4, 10, 20, and 30 mg/ml at initial sucrose-LDH solutions are denoted as 1.1, 2.9, 5.7, and 8.6 g-sucrose/g-LDH samples, respectively.

For all freeze-dried samples, LDH did not lose activity during preparation. The residual activity of freeze-dried LDH during storage is shown in Fig. 3.2. Ordinate values indicate residual activity of LDH, that is, relative values to the initial activity before storage.

For LDH alone and crystalline samples, LDH was remarkably inactivated during the storage. Compared with them, the stabilizing effect of sucrose for amorphous samples shows strong sucrose content dependency. LDH is most stabilized in the 2.9 g-sucrose/g-LDH sample. It is also stabilized in the 1.1 g-sucrose/g-LDH sample. The stabilizing effect is weak for the 5.7 g-sucrose/g-LDH sample. No stabilizing effect is noted for the 8.6 g-sucrose/g-LDH sample.

3. 3. 2 Results of analyses of XRD and DSC

XRD patterns before and during storage are shown in Fig. 3.3. Sucrose is kept amorphous during storage in the 1.1 and 2.9 g-sucrose/g-LDH samples. In contrast, crystallization of sucrose occurs in the 5.7 g-sucrose/g-LDH sample after 1 day storage, and in the 8.6 g-sucrose/g-LDH sample after 12 h storage. Thus the reduction of the stabilizing effect of sucrose for these samples should be due to the crystallization.

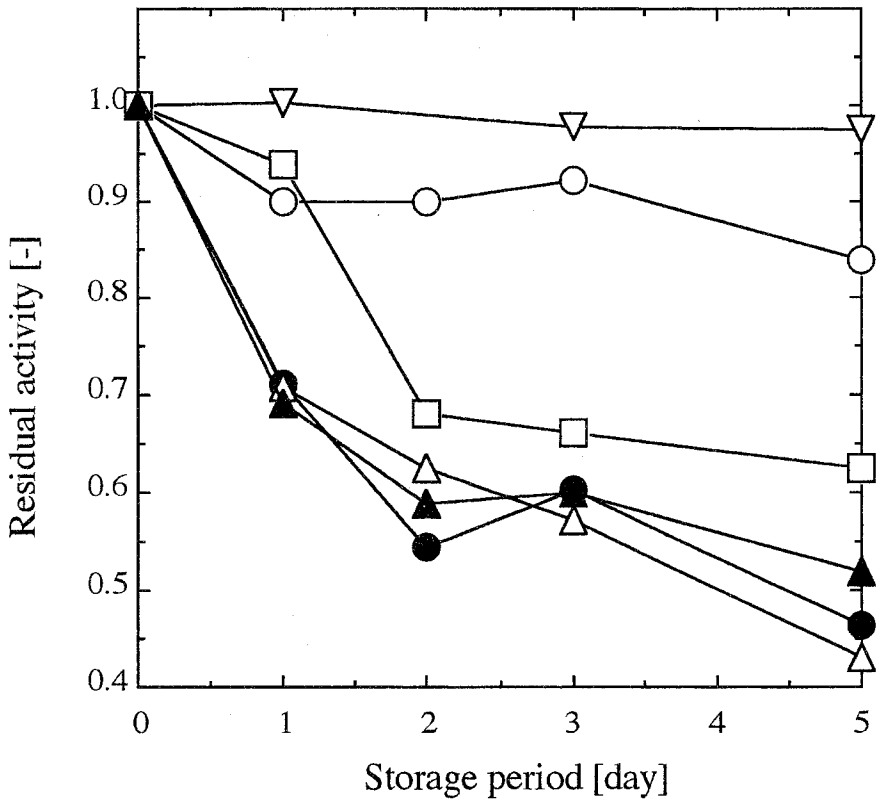


Fig. 3.2 Residual activity of LDH in (●) LDH alone sample, (▲) crystalline sample, and amorphous samples. Sucrose contents of amorphous samples were (○) 1.1, (▽) 2.9, (□) 5.7, and (△) 8.6 g/g-LDH.

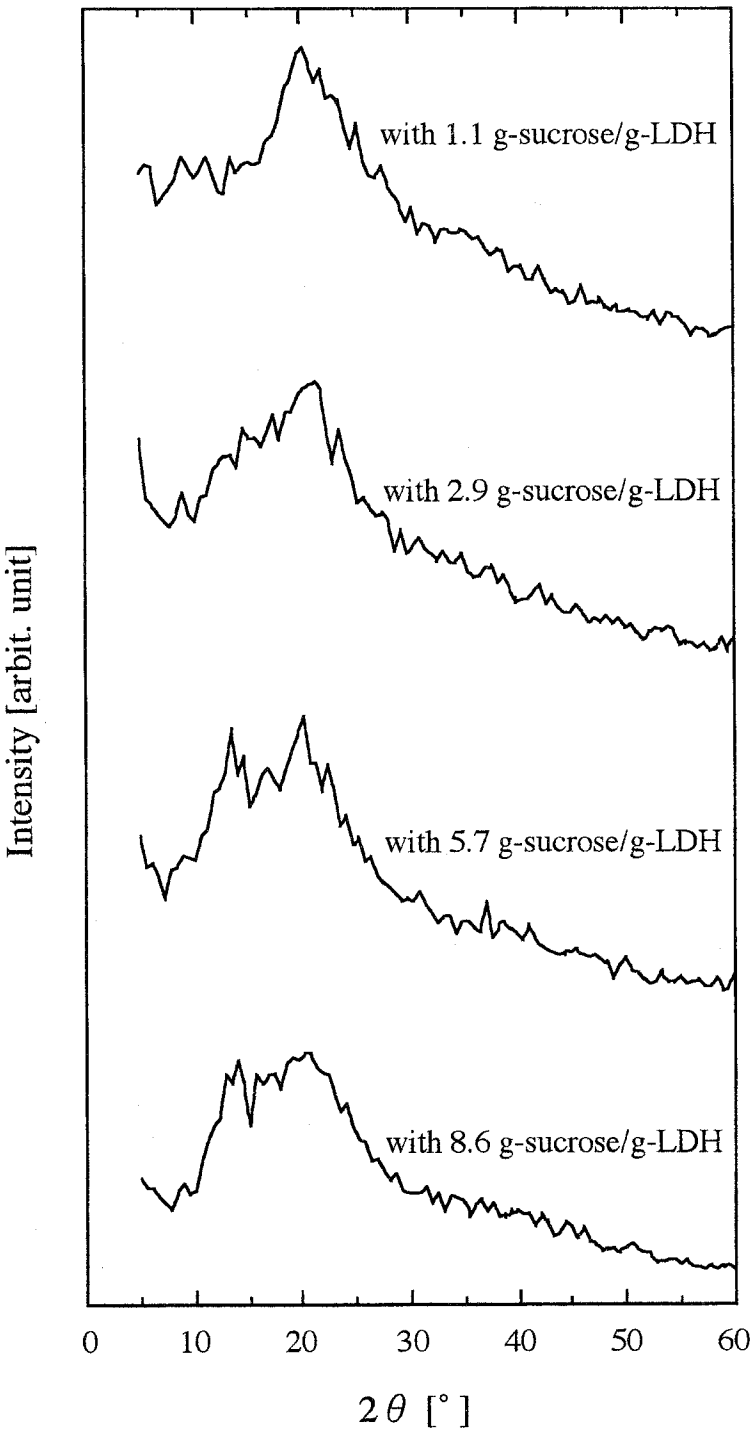


Fig. 3.3(a) XRD patterns of amorphous samples before storage

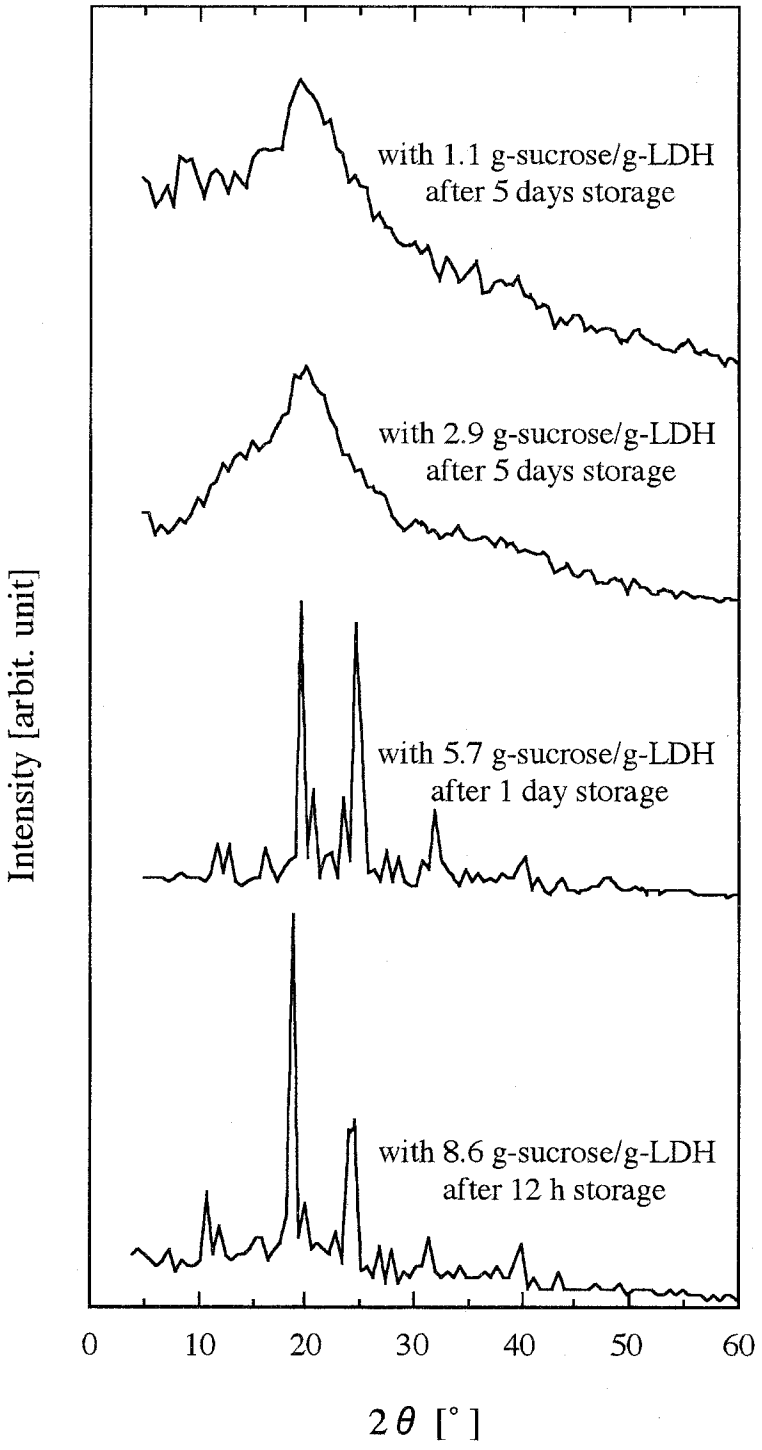


Fig. 3.3(b) XRD patterns of amorphous samples during storage

The difference in crystallization indicates that the physical stability of the amorphous structure strongly depends on the sucrose content. In general, we can evaluate the physical stability of amorphous structure with T_g. Then T_g was measured for all samples by DSC. Results are listed in Table 3.1. T_g of amorphous sucrose alone is also listed. T_g decreased with sucrose content. Moreover, T_g of the amorphous sucrose alone is the smallest. These results suggest that LDH stabilizes the amorphous structure of sucrose. The decrease of T_g should be caused by the decreasing stabilizing effect of LDH with increasing sucrose content.

Table 3.1 T_g of amorphous sucrose in amorphous samples

sucrose content [g-sucrose/g-LDH]	T_g [°C]
1.1	44.5
2.9	42.9
5.7	37.0
8.6	35.7
sucrose alone	27.9

3. 3. 3 Results of investigation of sucrose-LDH hydrogen bond

It is known that, with an increase in hydration levels of proteins, amide I and amide II bands of the IR spectra shift to lower and higher wavenumbers, respectively (Careri et at., 1979; Poole and Finney, 1984). Such alterations are also induced by the formation of sugar-protein hydrogen bond (Carpenter and Crowe, 1988, 1989). Hence the author evaluate the formation of hydrogen bond between sucrose and LDH by FT-IR measurement.

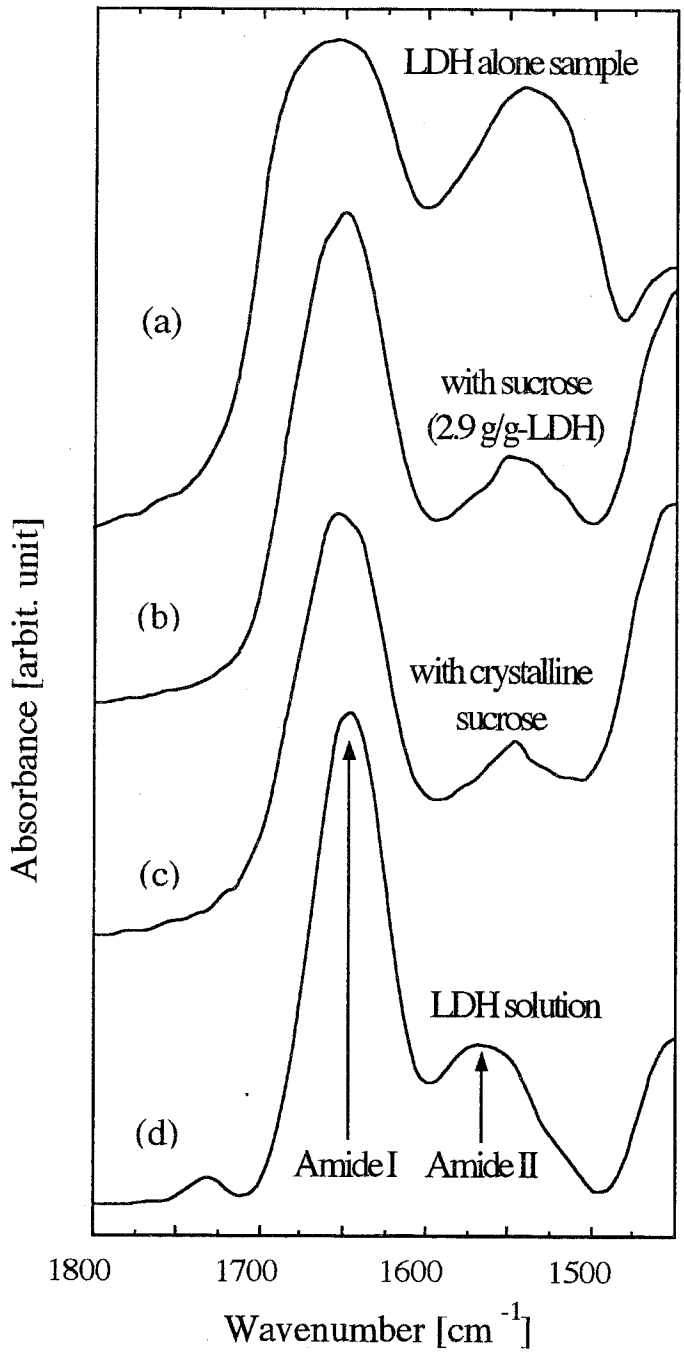


Fig. 3.4 IR spectra in the amide band region for (a) LDH alone sample, (b) amorphous sample with 2.9 g-sucrose/g-LDH, (c) crystalline sample, and (d) 7.0 mg/ml LDH solution

FT-IR spectra of the amide band region of some samples are shown in Fig. 3.4. Amide I and amide II bands are located respectively around 1650 cm^{-1} and 1550 cm^{-1}, and the wavenumbers of their peaks vary with the sample. From such spectra, the wavenumbers for the peaks of amide bands are determined.

3. 3. 3. 1 Differences in hydrogen bond formation for samples before storage

Figure 3.4 shows wavenumbers for the peaks of amide I and amide II bands. For LDH alone sample (indicated by the keys at 0 g/g-LDH sucrose content), wavenumbers are 1653 cm^{-1} for amide I and 1538 cm^{-1} for amide II, respectively. Compared with them, LDH in aqueous solution shows a remarkable low value of 1646 cm^{-1} for amide I and a high value of 1565 cm^{-1} for amide II because of hydration. The crystalline sample shows almost the same value of 1655 cm^{-1} for amide I and low value of 1545 cm^{-1} for amide II compared with the LDH alone sample. This result suggests that LDH in the crystalline sample is hydrated only slightly.

All the amorphous samples shows lower wavenumbers (1648-1649 cm^{-1}) for amide I and higher wavenumbers (1549-1551 cm^{-1}) for amide II than the crystalline sample. As described in section 3.3.4, such high hydration of LDH should be caused by sucrose-LDH hydrogen bond. The difference in hydrogen bond formation for the amorphous and the crystalline samples seems to be caused because amorphous sucrose has more structural flexibility than sucrose crystal, and thus sucrose molecules form hydrogen bonds with LDH more easily in the amorphous samples than in the crystalline sample.

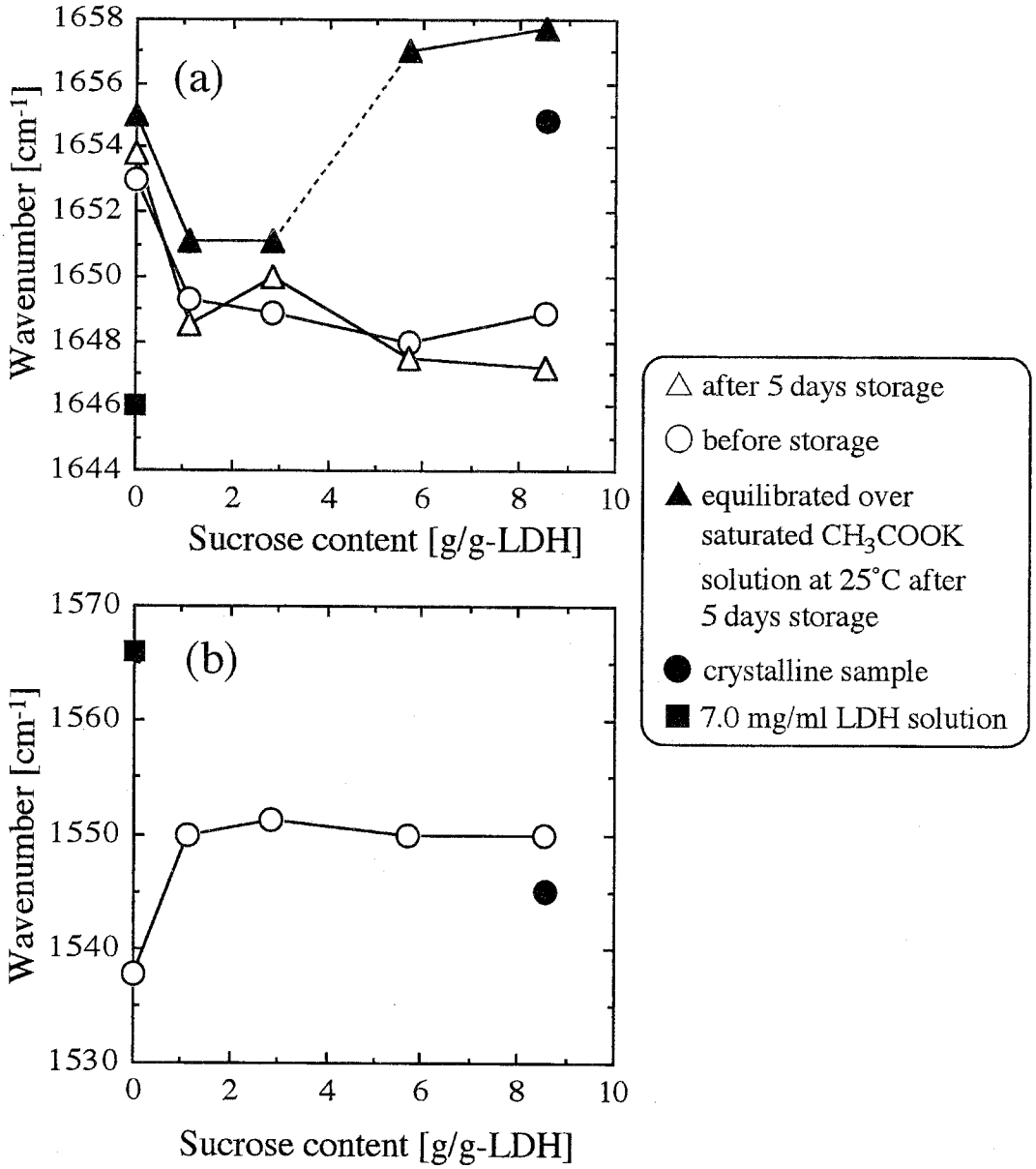


Fig. 3.5 Wavenumbers of the peaks for (a) amide I band and (b) amide II band of LDH alone samples and amorphous samples

The wavenumbers of amide bands for the amorphous samples are almost constant and independent of the sucrose content. This suggests that sucrose-LDH hydrogen bonds are almost fully formed for 1.1 g/g-LDH sucrose content, and that further addition of sucrose hardly yields further hydrogen bond formation.

3. 3. 3. 2 Change of hydration of LDH after 5 days storage

As described in section 3.3.2, crystallization of sucrose occurs in the 5.7 and 8.6 g-sucrose/g-LDH samples during storage. The phase transition is expected to influence the hydration of LDH, thus the change in hydration after 5 days storage is investigated. For this purpose, the author treated only amide I, because amide II was found to split into many unassigned bands during storage.

From Fig. 3.5(a), the 1.1 and 2.9 g-sucrose/g-LDH samples show low wavenumbers of the peak of amide I whether samples were re-equilibrated over saturated CH_3COOK solution for 5 days (see section 3.2.5) or not. Thus, in these samples sucrose-LDH hydrogen bond seems to be maintained even after 5 days storage. On the other hand, for the 5.7 and 8.6 g-sucrose/g-LDH samples, wavenumbers of the peak of amide I are altered drastically by the re-equilibration. When the samples are not re-equilibrated, they show remarkably low wavenumbers, which indicates high hydration of LDH. In contrast, when samples are re-equilibrated, the wavenumbers are rather high, which indicates low hydration of LDH. Such alterations seem to be caused as follows: when sucrose was crystallized, most of the sucrose-LDH hydrogen bonds are broken. At the same time water molecules involved in amorphous sucrose are released, and they form hydrogen bond with LDH. Therefore, when samples are not re-equilibrated, wavenumbers of amide I are quite low in spite of the serious loss of sucrose-LDH hydrogen bond. On the other hand, when the samples are re-equilibrated, most of the water-LDH

hydrogen bonds generated by crystallization are lost. Consequently, after re-equilibration, hydration of LDH decreases drastically and wavenumbers vary.

From the results of FT-IR analysis and the thermal stabilizing effect of sucrose, it is found that there is a close relationship between the stabilizing effect of sucrose and the sucrose-LDH hydrogen bond. In the case where sucrose-LDH hydrogen bond are formed and maintained during storage, LDH are stabilized remarkably. In contrast, without sucrose-LDH hydrogen bond formation, LDH is inactivated. Moreover, for the amorphous samples of 5.7 and 8.6 g-sucrose/g-LDH, many sucrose-LDH hydrogen bonds seem to be replaced by water-LDH hydrogen bonds, and the thermal stabilizing effect for these samples are inferior to that for the 1.1 and 2.9 g-sucrose/g-LDH samples. Thus we can consider that the thermal stabilizing effect of sucrose is caused by the sucrose-LDH hydrogen bond.

3. 3. 4 Results of the moisture content analysis

As described above, FT-IR analysis indicates high hydration of LDH in amorphous sucrose. However, both sucrose and water molecules can form hydrogen bonds with LDH. It has not been clarified which of the two forms hydrogen bonds in the amorphous samples. In order to discuss this problem, the author investigated the quantity of water by Karl-Fischer moisture titration.

In Table 3.2, equilibrium moisture contents of the amorphous samples at RH 23% are summarized. In order to estimate the quantity of water hydrating to LDH, the author estimated LDH-based water content W_{LDH}, or the water quantity of the samples which involve 1 g of LDH, from the data in Table 3.2. Results are shown in Fig. 3.6. We can see that W_{LDH} increased linearly with sucrose content.

It must be noted that the W_{LDH} for the 1.1 g-sucrose/g-LDH sample was less than that for the LDH alone sample (indicated by the closed circle). This means that LDH in the 1.1 g-sucrose/g-LDH sample was less hydrated compared with the LDH alone sample. Moreover, the water quantity hydrating to LDH in amorphous sucrose can be evaluated by the intercept of the line in Fig. 3.6. The value is 0.026 g/g-LDH, which is much lower than the water quantity (0.11 g/g-LDH) for the LDH alone sample. Therefore the high hydration level of LDH in the amorphous samples indicated by FT-IR analysis should be due to sucrose-LDH hydrogen bond, not water-LDH hydrogen bond.

Table 3.2 Moisture content of freeze-dried samples equilibrated with saturated CH_3COOK solution (RH 23%) at 25 °C

sucrose content [g-sucrose/g-LDH]	moisture content [g/g-dry matter]
0	0.113
1.1	0.034
2.9	0.037
5.7	0.037
8.6	0.039

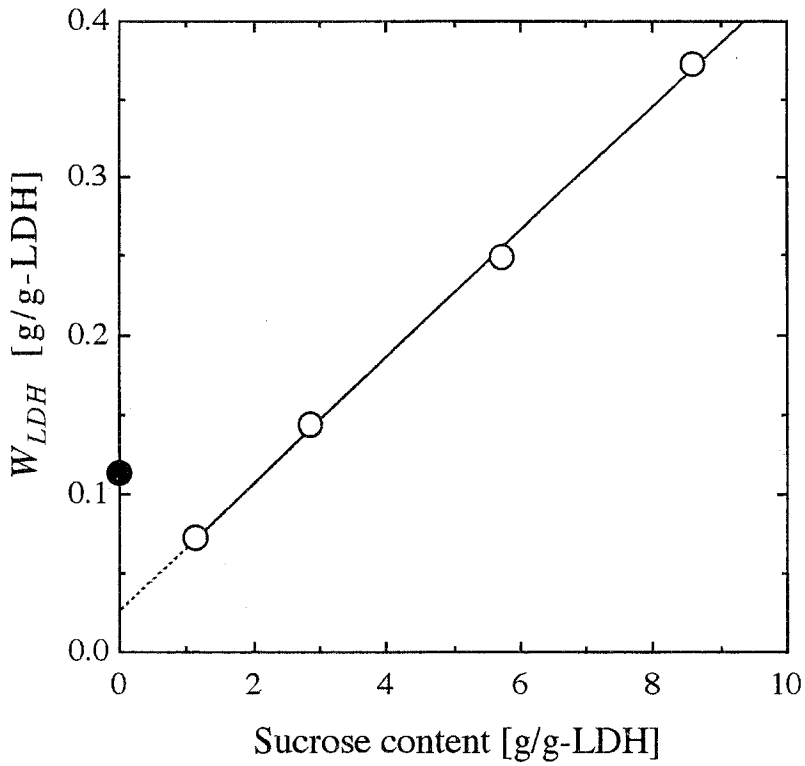


Fig. 3.6 LDH-based water quantity W_{LDH} as a function of sucrose content. Water quantity of (●) LDH alone sample is also shown.

3. 3. 5 Optimum sucrose content on stabilization of LDH

In Fig. 3.2, there is an optimum sucrose content for the thermal stabilizing effect of sucrose on LDH. The sucrose content dependency of LDH stability can be thought of as follows. The thermal stabilizing effect of sucrose increases with sucrose content. However, as shown in Fig. 3.3 and Table 3.1, as the sucrose content is higher, the crystallization of sucrose occurs more readily. Crystallization causes the loss of LDH-sucrose hydrogen bond, resulting in the loss of the stabilizing effect of sucrose. Consequently, the highest thermal stability of LDH is obtained at the highest sucrose content being able to maintain the amorphous structure during storage.

3. 3. 6 The thermal stabilizing effect of sugar on protein

On the basis of the results described above, the relation among the thermal stability of LDH, the crystallinity of sucrose, and the sucrose-LDH hydrogen bond is considered as shown in Fig. 3.7. The sugar-protein hydrogen bonds are formed more easily when the sugar added is amorphous than when the sugar is crystalline. Then the sugar-protein hydrogen bonds should support the higher order structure of protein and as the result, improve the thermal stability of the protein.

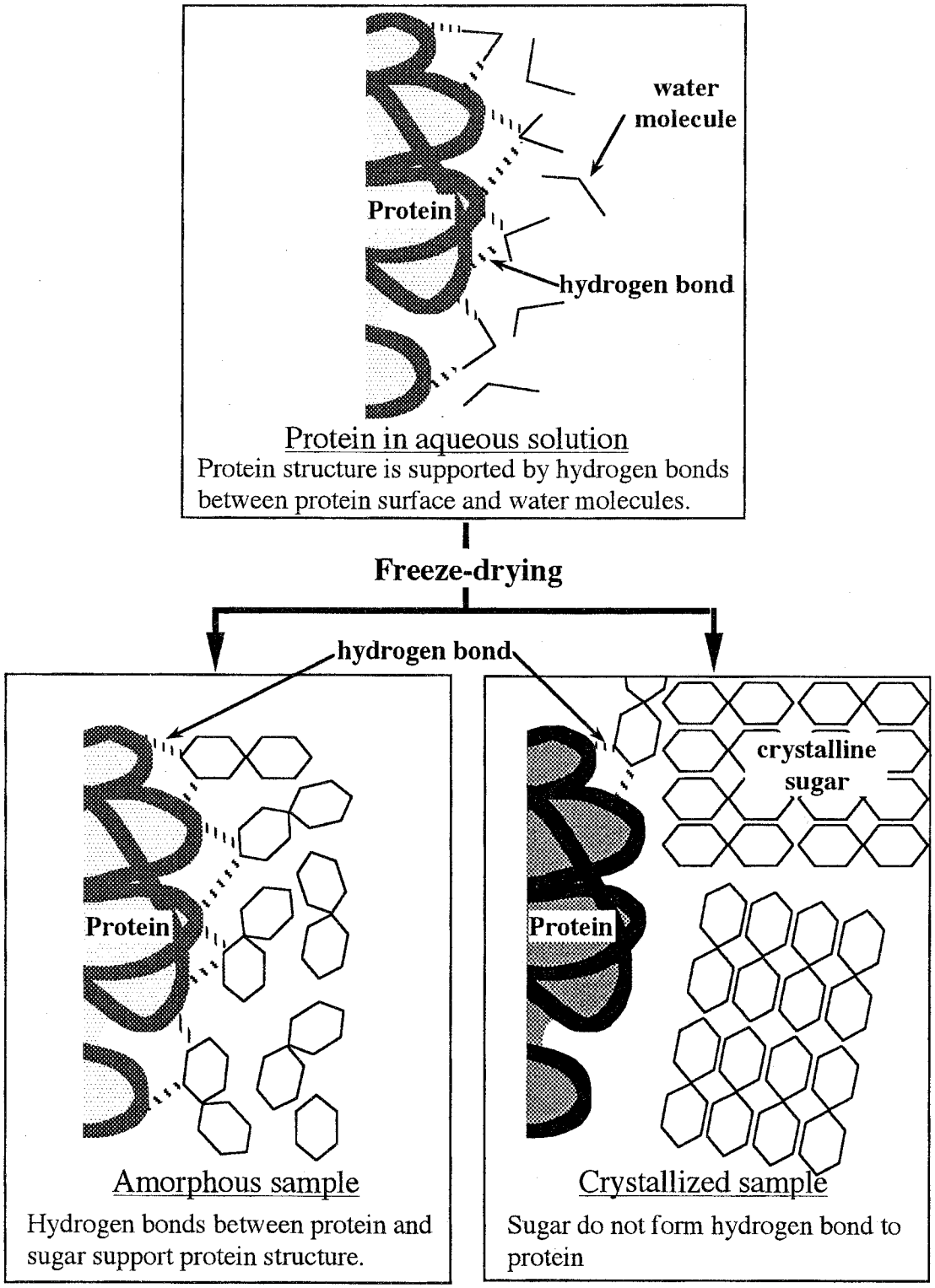


Fig. 3.7 Stabilizing effect of sugar on freeze-dried protein

3. 4 Conclusion

The author investigated the relation between the thermal stabilizing effect of sucrose on freeze-dried LDH and the degree of hydrogen bond formation between sucrose and LDH. It was found that the thermal stabilizing effect of sucrose was remarkable when the degree of sucrose-LDH hydrogen bond formation was high. From the result we can deduce that the thermal stabilizing effect of sugars on proteins closely relates to the sugar-protein hydrogen bonds. The author also studied the influence of sucrose content on the thermal stabilizing effect, and found that there was an optimum sucrose content for the stabilizing effect. The reason is that LDH protected amorphous structure of sucrose from crystallization that causes loss of sucrose-LDH hydrogen bonds. Thus, he can deduce that sugars and proteins work together to keep activities of proteins.

Chapter 4

Influences of Protein on Phase Transition of Amorphous Sugar

4. 1 Introduction

In the case where unstable materials are turned into products that are capable of being shipping and stored, they are often freeze-dried in the presence of sugars. In freeze-dried preparation, the labile component is embedded in the amorphous structure formed by sugar and, as a result, protected from degradation (Franks et al., 1991; Levine and Slade, 1992). However, the amorphous state is metastable, thus the crystallization of amorphous structure is likely to occur and cause quality defects (Makower and Dye, 1956; Tsouroufis et al., 1976; White and Cakebread, 1966). As an example, one can stabilize enzymes thermally by including them in amorphous structure of sugars, but the stabilizing effect of sugars is lost when sugars are crystallized as shown in Chapter 2. Because of such a close relationship between the amorphous structure of sugars and its stabilizing effect on labile components, it is important in the interest of keeping the quality of products to investigate the physical stability of amorphous structures of sugars.

Glass transition temperature (T_g) is usually used to evaluate the physical stability of amorphous structure of sugar. When the temperature of amorphous structure of sugar exceeds its T_g, the amorphous sugar turns into liquid like rubber, resulting in crystallization (Tsouroufis et al., 1976; To

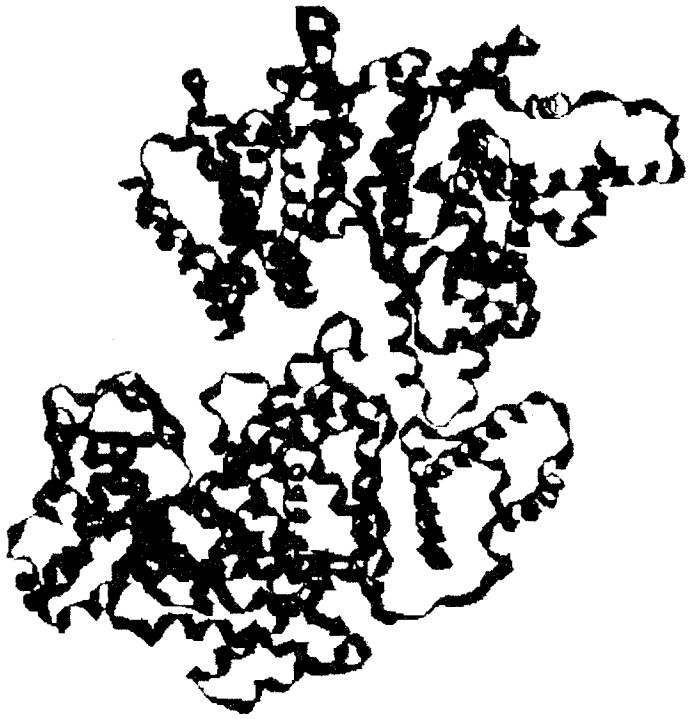
and Flink, 1978). It is known that T_g values of amorphous sugars are much influenced by moisture contents and coexisting components. Roos and Karel evaluated T_g values of amorphous sugars for a wide range of moisture contents and reported that T_g decreases as moisture content increases (1990, 1991a,b,c,d). Various components, such as carbohydrates, alcohols, salts, amino acids, are also found to influence T_g values (Orford et al., 1990; Roos and Karel, 1991a; te Booy et al., 1992).

According to these results, it is expected that the presence of protein also affects the physical stability of amorphous sugar because protein has many polarized residues on the surface. However, studies discussing such points have not yet been reported, although sugar-protein products are increasing in foods and pharmaceutical industry. Hence the author investigated the influence of protein on the amorphous structure of sugar. Amorphous sugar samples containing protein were prepared by freeze-drying. Sucrose, which is widely used for food products, was used in the present study. Bovine serum albumin (BSA) was used as a model protein, because it has been studied from various stands of views in detail. Samples were analyzed by differential scanning calorimetry (DSC); glass transition temperature T_g, crystallization temperature T_{cr}, melting temperature T_m, and enthalpies of crystallization and melting were determined. Moisture contents of samples were also measured by Karl-Fischer titration. The author discusses the influence of protein on the physical stability of amorphous sugar based on the results of analyses.

4. 2 Experimental

4. 2. 1 Preparation of amorphous sugar samples containing protein

Structure and characteristics of BSA are shown in Fig. 4.1. Sucrose (Nacalai Tesque Inc.) and BSA (Sigma Chemical Co.) were dissolved in distilled water to give sucrose concentration of 30 mg/ml and BSA concentration of 0–30 mg/ml. 5 ml of each test solution was transferred into a flask and frozen instantaneously with liquid nitrogen. Then the flask with sample was soaked in an ethanol bath, and the sample was freeze-dried at 1 Pa by three drying steps in order to keep the sample temperature below T_g and shorten the time required for sufficient dehydration; The sample was freeze-dried at -30°C for 24 h at the first step, at -10°C for 12 h at the second step, and at 0°C for 9 h at the third step. At the end of three drying steps, the residual moisture contents of sucrose samples without BSA were about 0.070, 0.050, and 0.025 g/g-sucrose. After further drying at room temperature for 1 h, water activity of the obtained samples was adjusted by the method described as follows: Water activity of samples was controlled to be zero by dehydrating thoroughly at 25°C in a vacuum desiccator with P_2O_5 (Nacalai Tesque Inc.) for few (at least two) days. Then samples were rehumidified at 25°C in a vacuum desiccator with saturated salt solutions (Greenspan, 1977) for about two weeks. Both dehydration and rehumidification of samples were confirmed to be completed for two days. In the present study three salts, that is, LiCl, CH_3COOK, and $MgCl_2$ solutions were 0.11, 0.23, and 0.33, respectively.



Human Serum Albumin [i)]

Bovine Serum Albumin [ii)]	
Molecular Weight	approx. 68000
Structure	1 subunits
Number of amino residues	607

Fig. 4.1 Bovine Serum Albumin
i) Carter and Ho (1994), ii) Dayhoff (1973)

4. 2. 2 Analysis of samples

It is known that the structural enthalpy of amorphous structure coagulated in thermodynamically unstable state decreases due to aging during storage below T_g (Fox and Flory, 1950; Tant and Wilkes, 1981). This enthalpy relaxation retards thermal response of the structure, resulting in over-estimation of T_g in DSC analysis. Thus, in order to obtain accurate T_g, DSC analyses were done as follows. At first 1-3 mg of samples were transferred into 20 μl aluminum pans in a nitrogen atmosphere and sealed hermetically to keep the moisture content constant. Then each sample was prewarmed to a temperature at least 10°C higher than its T_g value in order to eliminate the structural enthalpy relaxation. Similar procedures have been used by other researchers (Roos and Karel, 1991a; te Booy et al., 1992). After prewarming, each sample was scanned at a rate of 5°C/min from a temperature of at least 50°C lower than T_g to 180°C, using an empty aluminum pan as a reference. A Rigaku DSC TAS-200 was used for the measurement. Calibrations for temperature and heat flow were made with distilled water (T_m = 0.0°C, ΔH_m = 333 J/g) and indium (T_m = 156.6°C, ΔH_m = 28.45 J/g). From the obtained thermograms, T_g, T_{cr}, and T_m was determined as the onset temperatures of shifts in apparent specific heat due to transitions, and enthalpies due to crystallization (ΔH_{cr}) and melting (ΔH_m) were also determined from the peak area.

Moisture contents of sucrose-BSA samples were determined by using the Karl-Fischer titrimetric method. A Kyoto Electronics Karl-Fischer Titrator MK210 was used for the measurement.

4. 3 Results and Discussion

4. 3. 1 Moisture content

Moisture contents of samples at three water activities are listed in Table 5.1. The author did not measure moisture contents of sucrose alone sample and 0.067 g-BSA/g-sucrose sample at water activity 0.33 because sucrose crystallized fully during the rehumidification before the moisture contents reached equilibrium values.

Table 4.1 Moisture contents of amorphous sucrose samples containing BSA

BSA content	moisture content (g/g-dry matter)		
(g/g-sucrose)	aw=0.11	aw=0.23	aw=0.33
0	0.021	0.040	-[a]
0.067	0.020	0.037	-[a]
0.117	0.020	0.037	0.068
0.167	0.020	0.036	0.066
0.237	0.022	0.035	0.063
0.333	0.021	0.037	0.059
0.500	0.024	0.040	0.058
0.667	0.025	0.042	0.059
1.000	0.027	0.044	0.062
BSA alone	0.034	0.058	0.075

[a] not measured.

In Table 4.1 it is natural that moisture content increases with water activity, whereas the dependence of moisture content on BSA content is quite unique. With increasing BSA content, the moisture content decreases at low BSA content and increases at high BSA content.

In order to consider what happens, the author has calculated moisture content W_s [g-water/g-sucrose] from the data in Table 4.1 and W_s is plotted against BSA content in Fig. 4.2. From Fig. 4.2, it is found that W_s is described with two lines that crossed at BSA content 0.17, 0.23, and 0.42 g/g-sucrose at water activities 0.11, 0.23, and 0.33, respectively.

In Fig. 4.2, the value of slope has the dimension of [g-water/g-BSA] and is identical to moisture content of BSA basis. Thus it is worth comparing values of the slope with the moisture contents for the samples of BSA alone listed in Table 4.1. Here, the slopes are 0.036, 0.058, and 0.076 for water activities 0.11, 0.23, and 0.33, respectively. These values of the slopes agree well with the moisture contents for the samples of BSA alone. On the other hand, for the lines for lower BSA content, values of slope are 0.016, 0.017, and 0.016 for water activities 0.11, 0.23, and 0.33. These values are less than the moisture contents in Table 4.1.

From these results we can deduce two things: (1) For BSA content less than about 0.4 g/g-sucrose, BSA with sucrose has less hydrated water molecules than BSA without sucrose. Such situation could occur because BSA included in sucrose forms hydrogen bonds with hydroxyl groups of sucrose molecules. In fact, the formation of sugar-protein hydrogen bonds in freeze-dried materials is reported in literature (Carpenter and Crowe, 1989; Crowe et al., 1990), and also in the chapter 3. (2) The amount of BSA embedded in 1 g of sucrose is up to about 0.17, 0.23, and 0.42 g/g-sucrose at water activities 0.11, 0.23, and 0.33, respectively. When BSA was added above that, the excess of BSA could not be embedded completely in amorphous matrix of sucrose, rather it was in a similar state of BSA in the sample of BSA alone.

Fig. 4.2 shows that the BSA content at the cross point of two lines increases with an increase of water activity. This result suggests that the amorphous sucrose can embed more BSA as water activity increases. The reason for this phenomenon is not obvious at the present stage, but the phenomenon is important for understanding the physical properties of amorphous sugars.

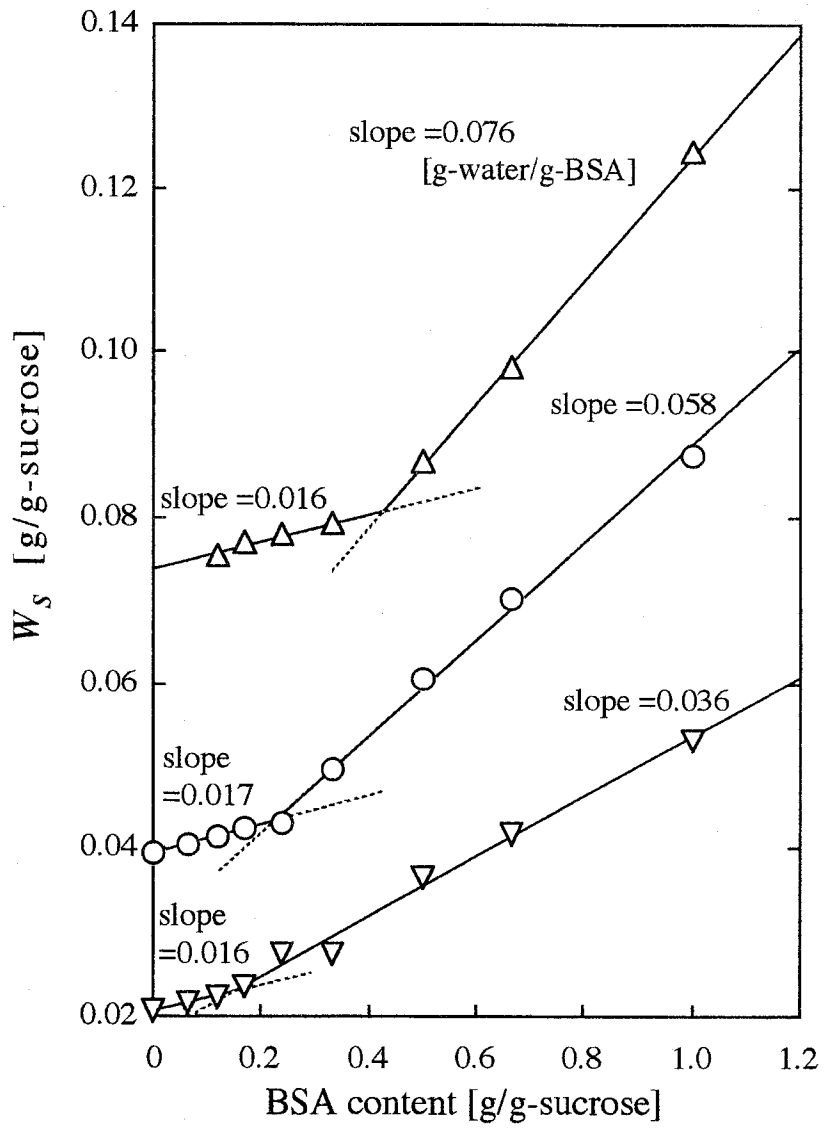


Fig. 4.2 Water quantities of samples for 1 g of sucrose. Water activities are 0.11 (▽), 0.23 (○), and 0.33 (△).

4. 3. 2 Differential scanning calorimetry analysis

DSC thermograms of amorphous sucrose samples containing BSA are shown in Fig. 4.3. The endothermic shift due to glass transition of amorphous sucrose is detected for all samples. For the samples whose BSA contents are up to 0.33 g/g-sucrose, crystallization and melting of sucrose were detected. As for the sucrose alone sample, corresponding experimental values reported in literature (Roos and Karel, 1991b) are 56.6±3.4, 104.4±2.3, and 183.5±1.2°C for T_g, T_{cr}, and T_m, respectively. One can see that the corresponding results shown in Fig. 4.3 agree well with the values reported. Thermograms of the samples whose BSA contents are equal to or more than 0.50 g/g-sucrose show slight endothermic peaks at 130°C and ascendance of the apparent specific heat at high temperature above 170°C, which may be caused by physico-chemical change of BSA. T_g calculated from these results are used in discussion in the following sections. T_{cr}, T_m, and enthalpies due to these phase transitions are also discussed below.

4. 3. 2. 1 Glass transition temperature

T_g for various contents of water and BSA are shown in Fig. 4.4. Roos and Karel (1991b) reported T_g values for solely sucrose samples humidified at various water activities. The values are 56.6±3.4, 37.8±8.0, and 27.9±2.4°C at water activities 0, 0.11, and 0.23, respectively. The results for sucrose alone samples shown in Fig. 4.4 agree well with their results.

From Fig. 4.4 one can see that T_g decreases with an increase in water activity. It is generally known that the viscosity of amorphous structure decreases drastically as the temperature of the sample increases above T_g. This drastic decrease is ascribed to the collapse of amorphous structure

(Roos and Karel, 1991d). Therefore a decrease in T_g means the weakening of amorphous, or reduction of intermolecular force that holds amorphous structure. This should happen when sucrose-sucrose hydrogen bonds decrease and sucrose-water hydrogen bonds increase as moisture content increases.

Addition of BSA raises or lowers the T_g values, depending on the water activity, and T_g approaches a constant value as BSA content increases. The degree of the change of T_g depends on the water activity of the sample. At water activity equal to 0, addition of BSA lowers T_g by 5°C lower than T_g for sucrose alone. In contrast, addition of BSA raises the T_g of rehumidified samples. Moreover, the degree of change of T_g by addition of BSA increases with water activity.

This unique dependence of T_g on BSA may be interpreted as follows. The author assumes that the influence of BSA on the stability of amorphous structure depends on the strength of interaction between amorphous sucrose and BSA relative to the intermolecular forces in amorphous sucrose (Fig. 4.5). Here, the "strength of intermolecular forces" is determined by the mass ratio of sucrose and water molecules in amorphous structure. If interaction between sucrose and BSA is weaker than the strength of intermolecular forces in amorphous sucrose, an increase in BSA content should weaken the whole amorphous structure, and thus T_g decreases. On the contrary, if interaction between sucrose and BSA is strong enough, an increase in BSA content raises T_g. The strength of intermolecular forces in amorphous sucrose depends on moisture contents. An increase in moisture contents weakens the amorphous structure. Therefore, the addition of BSA can weaken the amorphous structure at low moisture content and strengthen it at high moisture content.

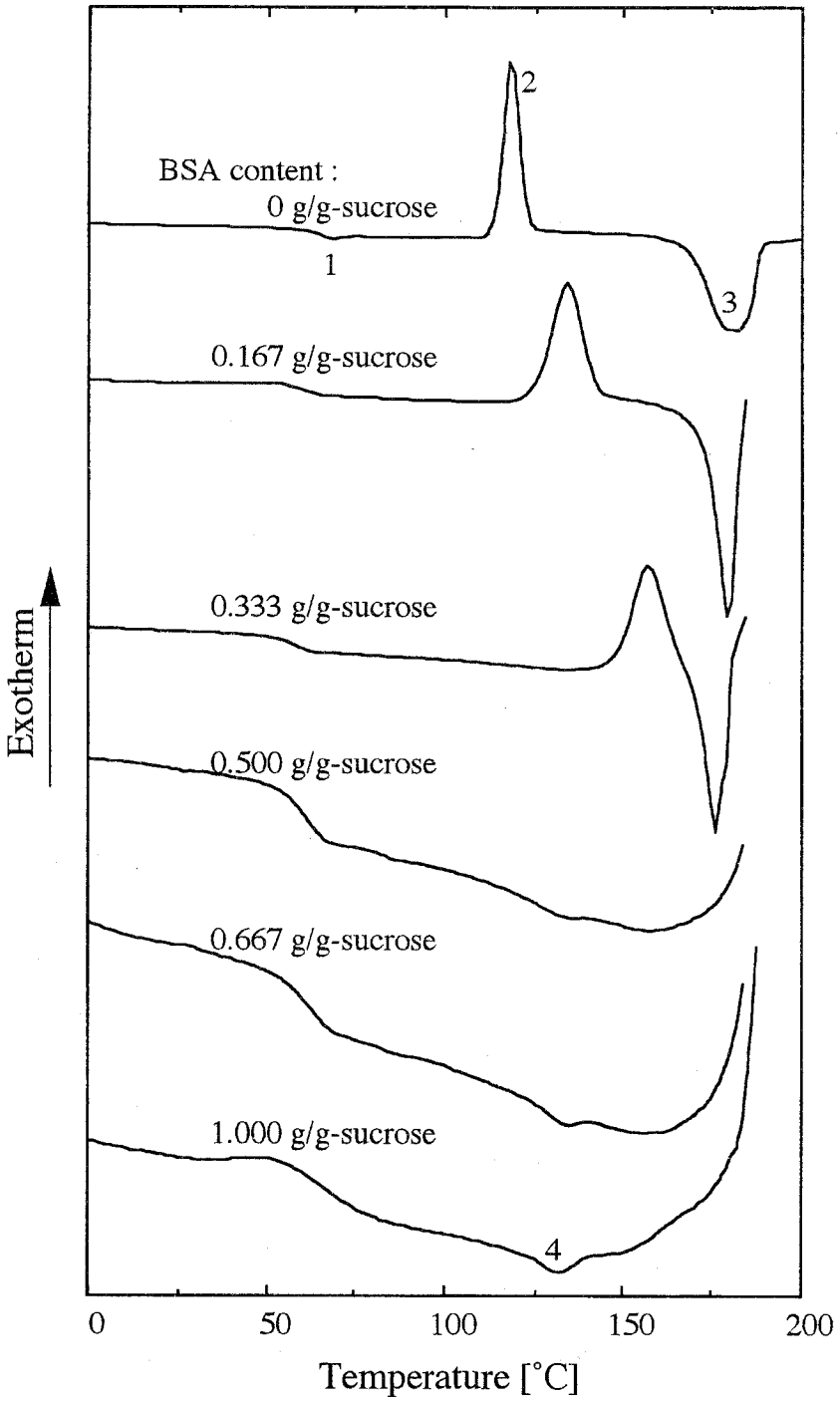


Fig. 4.3(a) DSC thermograms of amorphous sucrose dehydrated thoroughly at water activity 0. The thermograms show glass transition (1), crystallization (2), melting (3), and endothermic peak (4) that may be due to physico-chemical change of BSA.

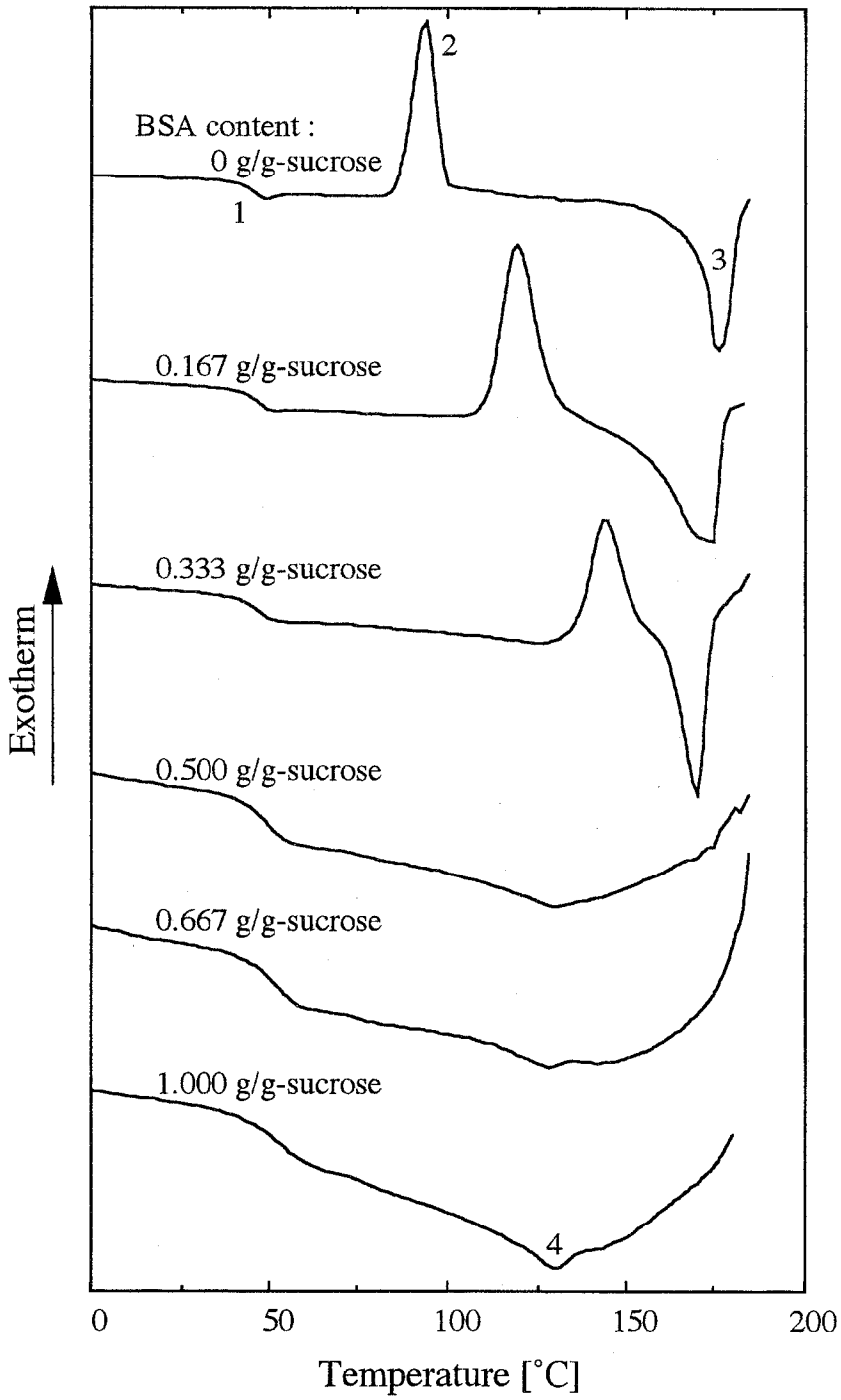


Fig. 4.3(b) DSC thermograms of amorphous sucrose equilibrated at water activity 0.11. The thermograms show glass transition (1), crystallization (2), melting (3), and endothermic peak (4) that may be due to physico-chemical change of BSA.

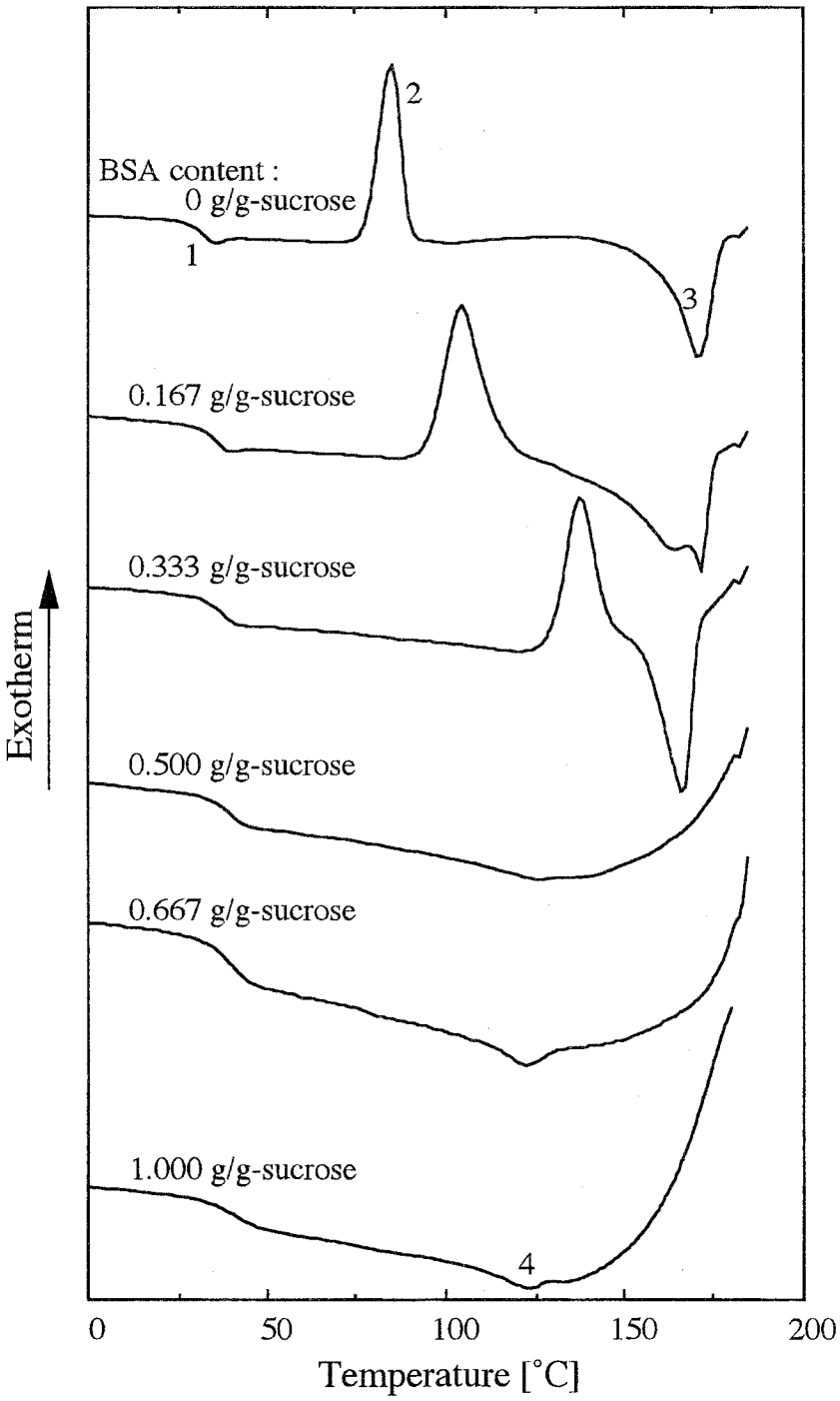


Fig. 4.3(c) DSC thermograms of amorphous sucrose equilibrated at water activity 0.23. The thermograms show glass transition (1), crystallization (2), melting (3), and endothermic peak (4) that may be due to physico-chemical change of BSA.

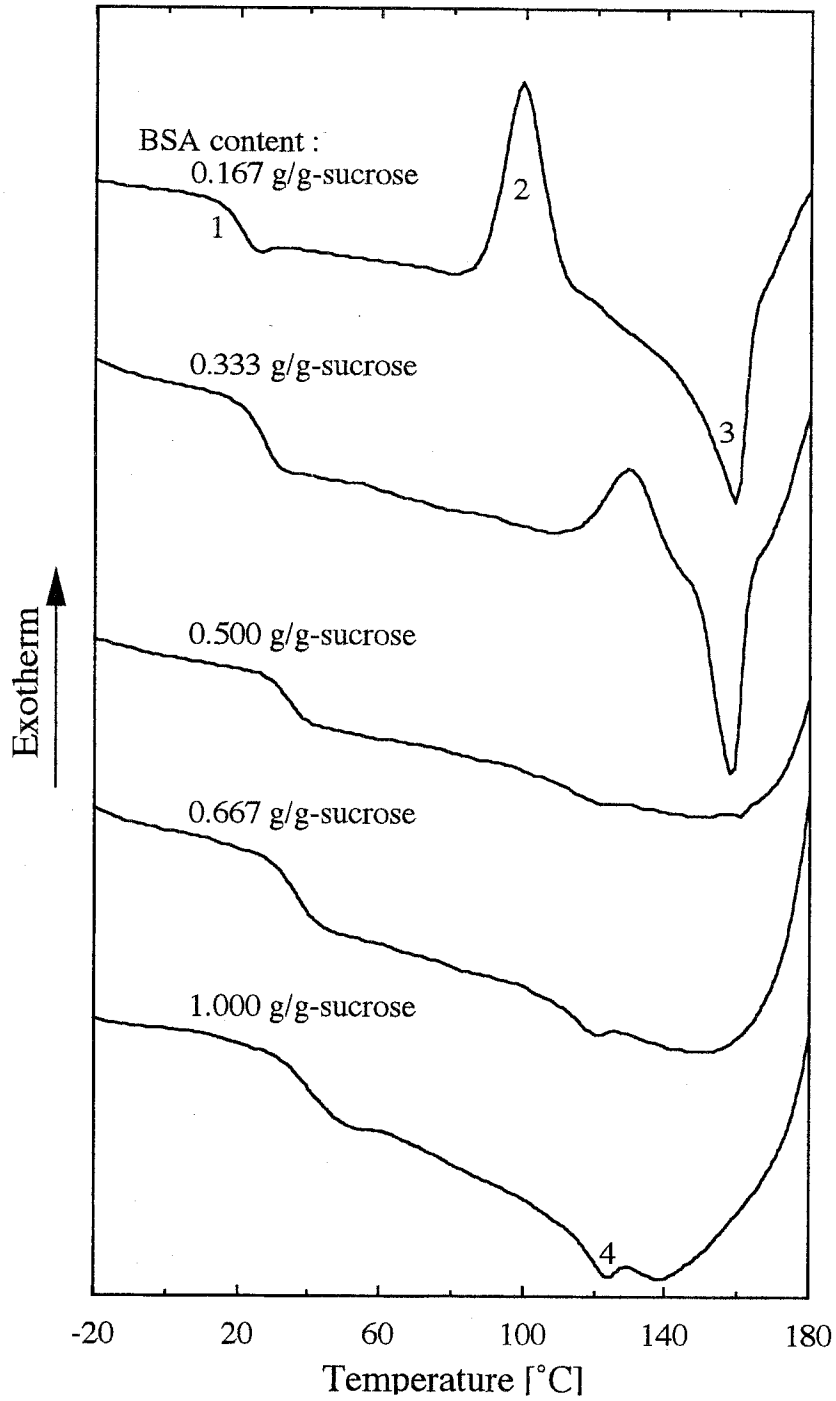


Fig. 4.3(d) DSC thermograms of amorphous sucrose equilibrated at water activity 0.33. The thermograms show glass transition (1), crystallization (2), melting (3), and endothermic peak (4) that may be due to physico-chemical change of BSA.

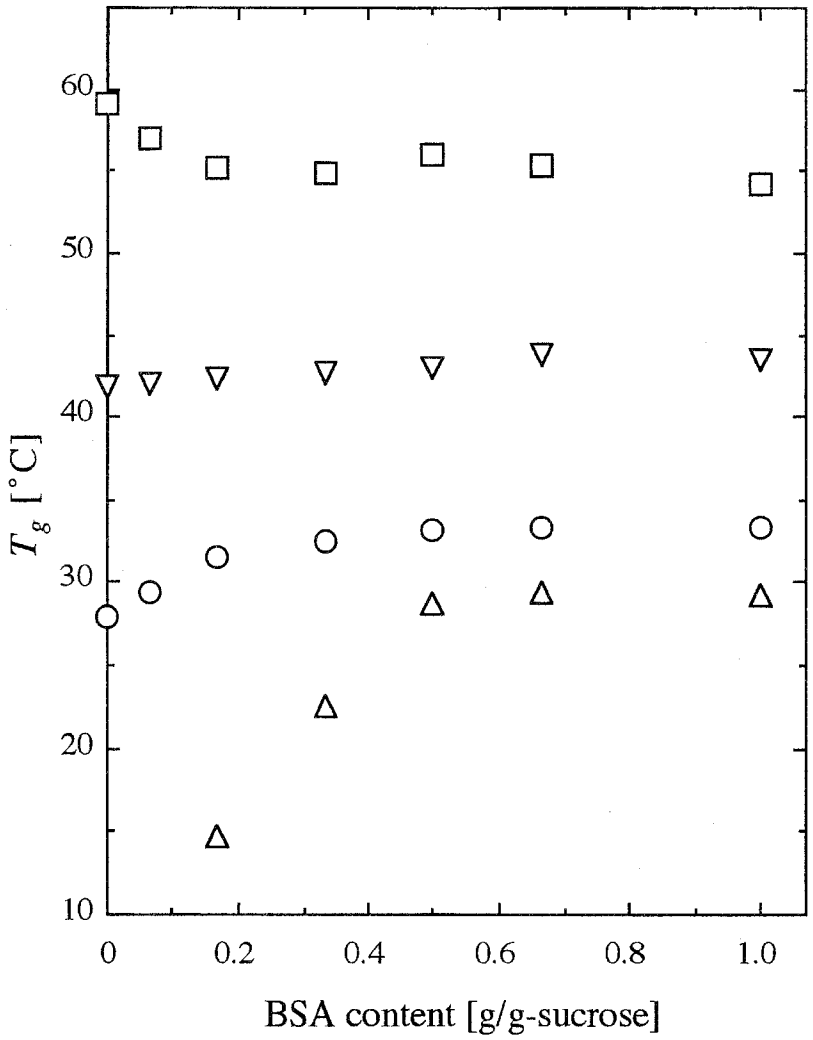


Fig. 4.4 T_g of amorphous sucrose for various contents of BSA. Water activities are 0 (□), 0.11 (▽), 0.23 (○), and 0.33 (△).

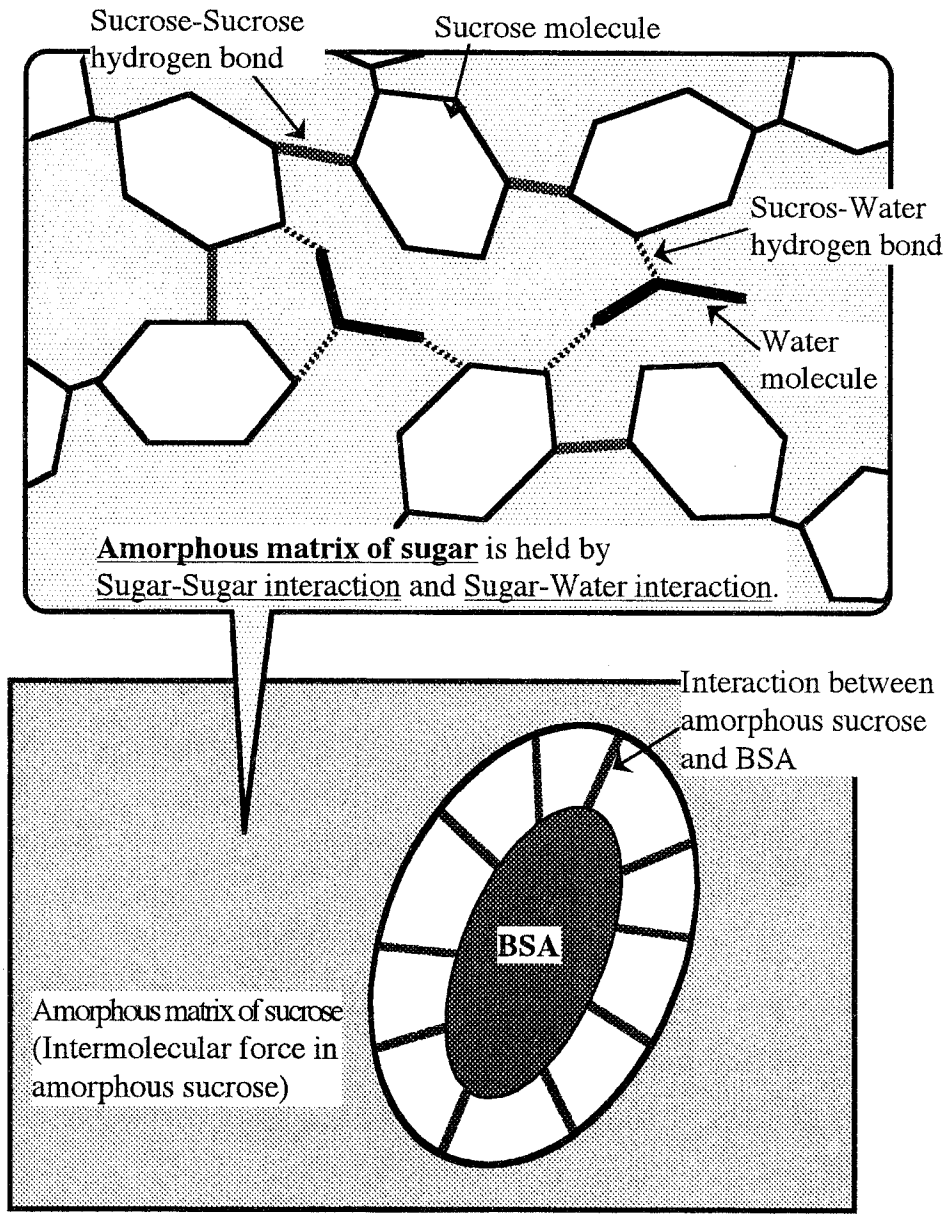


Fig. 4.5 Interactions holding amorphous structure of sucrose in freeze-dried sucrose-BSA mixture.

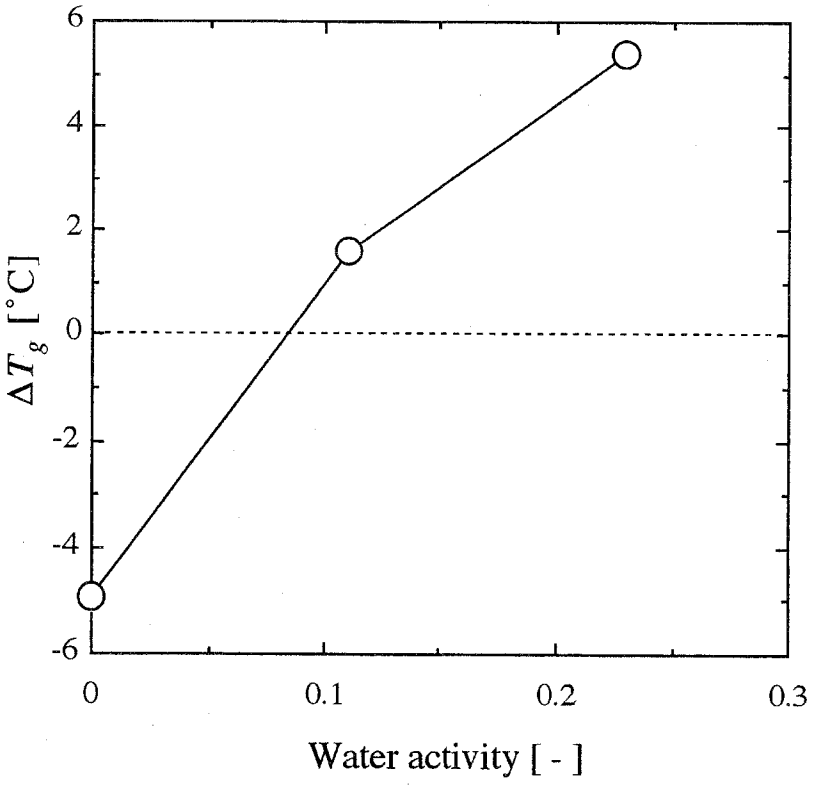


Fig. 4.6 The dependence of ΔT_g on water activity. ΔT_g is the difference between theT_g value of the sample without BSA and that of the sample whose BSA content is 1.0 g/g-sucrose.

In order to compare the strength of sucrose-BSA interaction with the strength of intermolecular forces in amorphous sucrose, the author has plotted ΔT_g, a difference between the T_g value of sucrose alone and that of the sample whose BSA content is 1.0 g/g-sucrose, as a function of water activity in Fig. 4.6. When ΔT_g takes negative or positive value, the strength of sucrose-BSA interaction is weaker or stronger than the strength of intermolecular forces in amorphous sucrose. When ΔT_g is equal to 0, the strength of sucrose-BSA interaction and the strength of intermolecular forces are equivalent. One can see such situation occurring when water activity is about 0.1.

4. 3. 2. 2 Crystallization and melting

In Fig. 4.7 T_{cr} and T_m are plotted against BSA content. As described in the above section, peaks due to crystallization and melting of sucrose are not detected for samples of BSA content equal to or more than 0.5 g/g-sucrose. As for sucrose alone samples, T_{cr} and T_m values were reported by Roos and Karel (1991b). The T_{cr} values were 104.4±2.3, 83.7±7.6, and 75.1±4.1°C at water activities of 0, 0.11, and 0.23, respectively. Corresponding T_{cr} values obtained in the present study agree well with those T_{cr} values. T_m values reported by Roos and Karel were 183.5±1.2, 172.1±4.2, and 165±2.1°C at water activities of 0, 0.11, and 0.23, respectively. It must be noted that they defined T_m as the temperature at the top of the endothermic peak for melting, and that the definition is different from the one in the present study. As described in section 4.2.1, T_m is defined as the onset temperature of shift in apparent specific heat. Although there is such a difference in definition, T_m values of the present study are almost the same as those of Roos and Karel.

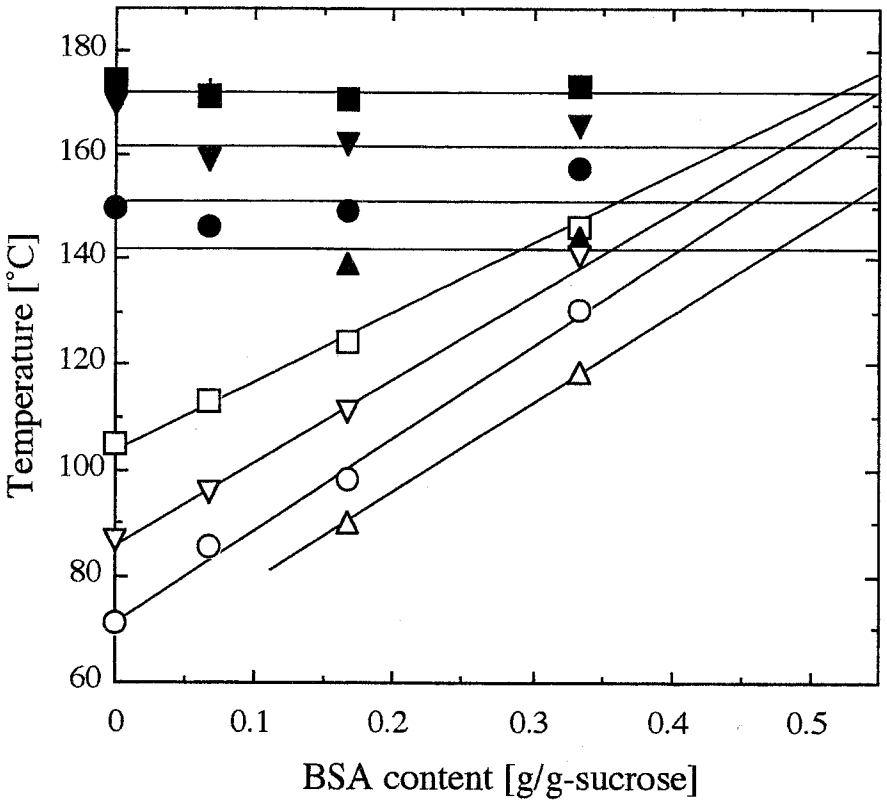


Fig. 4.7 BSA content dependence of T_{cr}(open key) and T_m (closed key). Water activities are 0 (□ and ■), 0.11 (▽ and ▼), 0.23 (○ and ●), and 0.33 (△ and ▲).

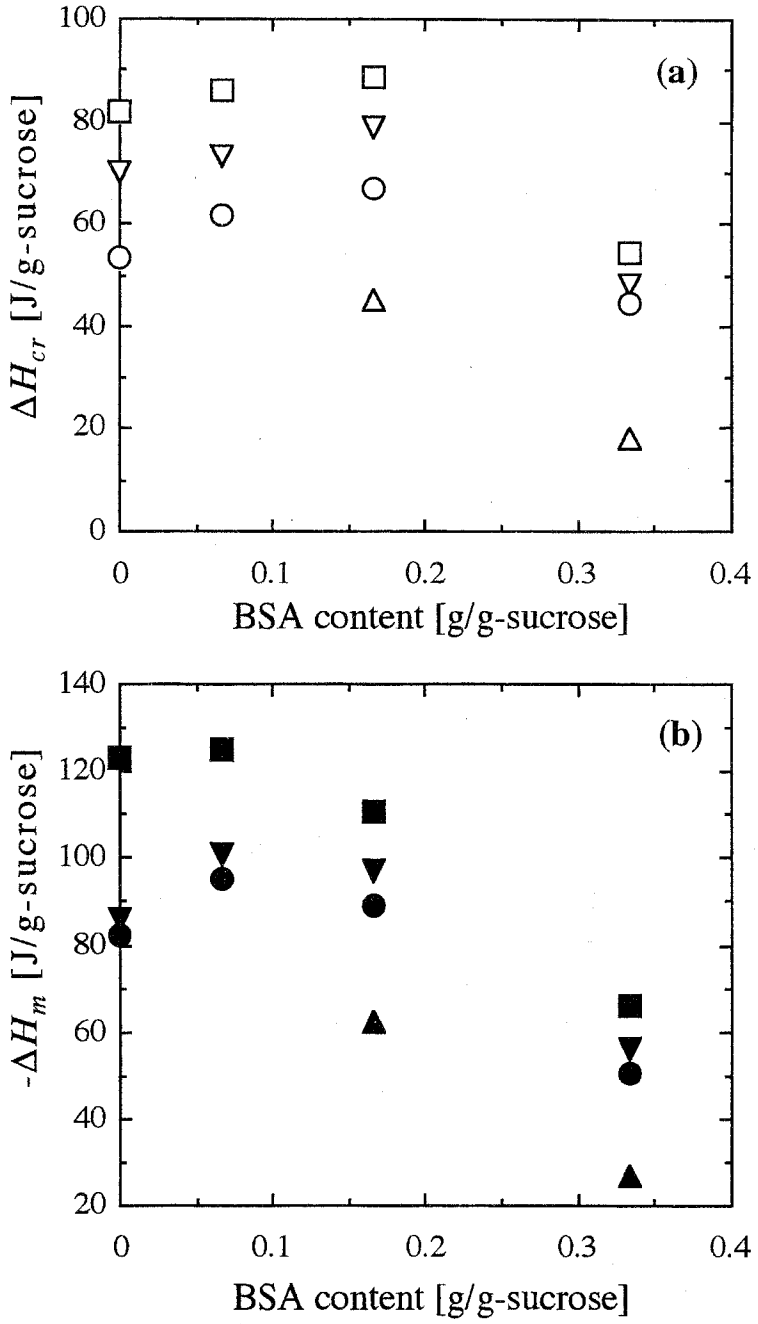


Fig. 4.8 BSA content dependence of ΔH_{cr}(a) and ΔH_m (b). Water activities are 0 (□ and ■), 0.11 (▽ and ▼), 0.23 (○ and ●), and 0.33 (△ and ▲).

Figure 4.5 shows that T_{cr} and T_m depended strongly on water activity. T_{cr} shows remarkable dependence on BSA content as well as water activity. T_{cr} increased linearly with BSA content. The remarkable ascendance of T_{cr} indicates that BSA should hinder crystallization of sucrose. On the other hand, T_m seems to be independent of BSA content.

As shown in Fig. 4.7, for each water activity the two lines representing T_{cr} and T_m crossed in the range of BSA content from 0.45 to 0.55 g/g-sucrose. This result seems to suggest that the addition of BSA more than about 0.5 g of pergram of sucrose causes disappearance of crystal state of sucrose.

In Fig. 4.8 enthalpies of crystallization (ΔH_{cr}) and melting (ΔH_m) of sucrose for various water activities and BSA contents are shown. With increase in BSA content, ΔH_{cr} and ΔH_m increased slightly at low BSA content ranging up to about 0.07 g/g-sucrose, and above that, decreased remarkably. The reason of the dependency of ΔH_{cr} and ΔH_m on BSA content is not obvious at the present stage, but the decrease in ΔH_{cr} and ΔH_m found above 0.07 g/g-sucrose may be caused because sucrose is hindered to crystallize with increasing BSA content.

4. 3. 2. 3 Complementary effect of sugar and protein

It is known that protein embedded in an amorphous matrix of sugar is protected from denaturation during dehydration and storage, while protein is not stabilized in crystallized sugar (Franks et al., 1991;Levine and Slade, 1992; Izutsu et al., 1991). On the other hand, in the present study, BSA embedded in amorphous sucrose is found to increase T_g except for the case where water activity of the sample is lower than 0.1. T_{cr} is found to increase significantly by addition of BSA at each water activity. From these results we can deduce that protein is effective in stabilizing an amorphous matrix of sugar and preventing it from crystallization. The presence of an

amorphous matrix of sugar thus stabilized promotes the stability of protein. This is because in the present case, thermal denaturation of protein is caused by the change of higher order structure of it, and the amorphous matrix of sugar stabilizes the higher order structure.

4. 4 Conclusion

In the present study, the author elucidated the influence of BSA on the physical stability of amorphous structure of sucrose by using DSC. The author has found that T_g increases with BSA content except for the case where water activity of the sample is extremely low. T_{cr} is found to increase with BSA content. This means that BSA contributes to stabilizing the amorphous structure of sucrose, and the stabilized amorphous structure of sugar promotes the stability of protein. From results of moisture content analyses, it was indicated that the hydration state of BSA embedded in the amorphous sucrose is lower than that of BSA alone sample. Based on the insight, the author evaluated the amount of BSA embedded in 1 g of sucrose.

Of course extensive work is needed in order to investigate whether the present result has generality or not, and such work will give us basic information which is helpful for preparation of food and drugs containing proteins.

Chapter 5

Glass Transition Behavior of Amorphous Sugar Containing Protein

5. 1 Introduction

In pharmaceutical industry, proteins that are feasible for therapeutic application but labile in aqueous system are often freeze-dried with sugars. In the freeze-dried preparations, proteins are embedded in the amorphous structures formed by sugars and protected from degradation (Franks et al., 1991; Levine and Slade, 1992; Izutsu et al., 1991). Glass transition of amorphous sugar strongly affects the stabilizing effect of them on proteins. Glass transition causes crystallization of sugar, resulting in the loss of the stabilizing effect of sugar on protein as shown in Chapter 2. Hence the information concerning such behavior of amorphous sugars is important for prediction and improvement of the storage stability of sugar-protein preparations.

Many studies have been reported on glass transition of amorphous sugars, whereas the influence of protein on the behavior has been rarely studied. In Chapter 4, the author investigated the influence of BSA on glass transition and found that addition of BSA lowered T_g of amorphous sucrose when water activity was 0, but raised it when water activity was above 0.11.

This result demonstrates that the influence of protein on T_g needs to be discussed for understanding the physical nature of sugar-protein mixture. It

is desirable to discuss the influence of protein for various amorphous sugars. Hence, in this study, the author investigated glass transition of sugar-protein samples for sucrose, trehalose, lactose, and maltose. BSA was used as a model protein. Sugar-BSA samples were obtained by freeze-drying sugar-BSA solutions. Moisture contents and T_g values of samples at several water activities ranging from 0 to 0.43 were measured by Karl-Fischer titration and differential scanning calorimetry (DSC), respectively. The author discusses the influence of BSA on glass transition of amorphous sugars.

5. 2 Experimental

5. 2. 1 Preparation of amorphous sugar containing BSA

Sucrose, trehalose, lactose, and maltose, were purchased from Nacalai Tesque Inc., and BSA was from Sigma Chemical Co. (St. Louis, U.S.A.). For each sugar, sugar-BSA solutions, whose sugar and BSA concentrations were 30 and 15 mg/ml respectively, were prepared with distilled water. Sugar solutions of 30 mg/ml and BSA solution of 15 mg/ml were also prepared. 5 ml of each solution was freeze-dried and then water activity of obtained samples was adjusted by saturated salt solutions in the way described in Chapter 4.

5. 2. 2 Analysis of samples

T_g and moisture contents of sugar-BSA samples obtained were analyzed as described in Chapter 4.

5. 3 Results and Discussion

5. 3. 1 Amorphous sugar samples

Prior to discuss the result of sugar-BSA samples, the author discusses the result of amorphous sugar samples.

5. 3. 1. 1 Moisture content of amorphous sugar samples

Sorption isotherms of moisture on freeze-dried sugars at 25°C are shown in Fig. 5.1. In Fig. 5.1, values in literature (Makower and Dye, 1956; Roos and Karel, 1991a, b) are also shown. Values in this study are well consistent with those reported by Makower and Dye. However, Fig. 5.1 shows discrepancies between values in this study and those reported by Roos and Karel. This seems because moisture contents in this study and reported by Makower and Dye are confirmed to be completely equilibrated to given water activities, whereas those reported by Roos and Karel are obtained after rehumidification for only one day and might be insufficiently equilibrated.

Fig. 5.1 shows that amorphous trehalose have the highest sorption affinity. Amorphous lactose and maltose are considered to have roughly equivalent affinity, and amorphous sucrose shows the lowest sorption affinity.

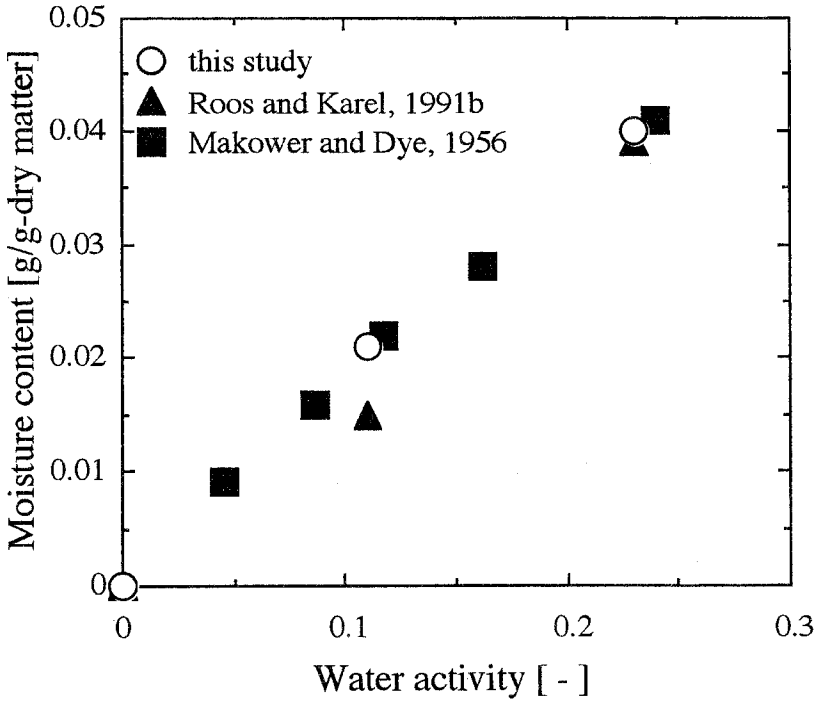


Fig. 5.1(a) Sorption isotherms of amorphous sucrose

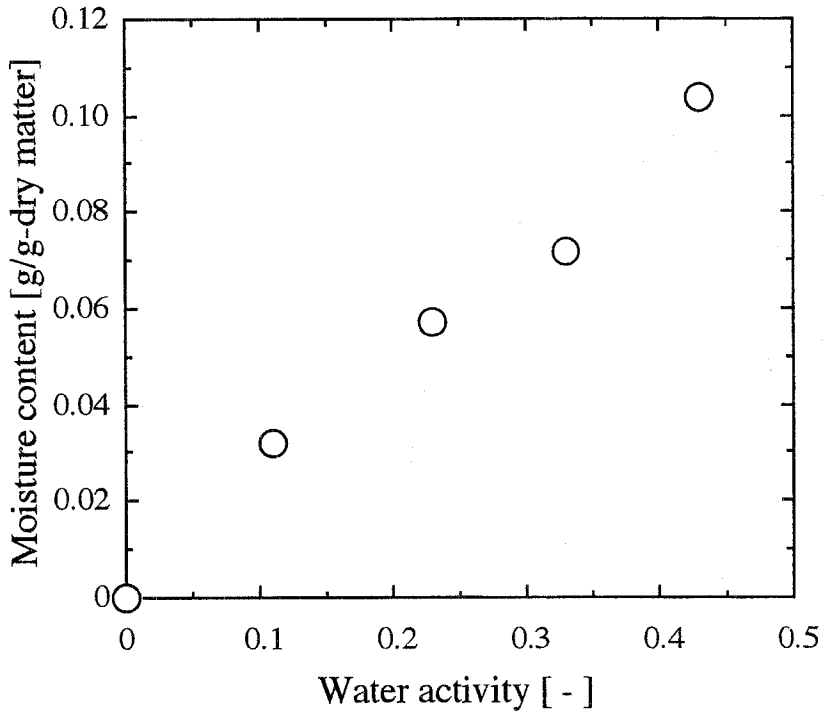


Fig. 5.1(b) Sorption isotherms of amorphous trehalose

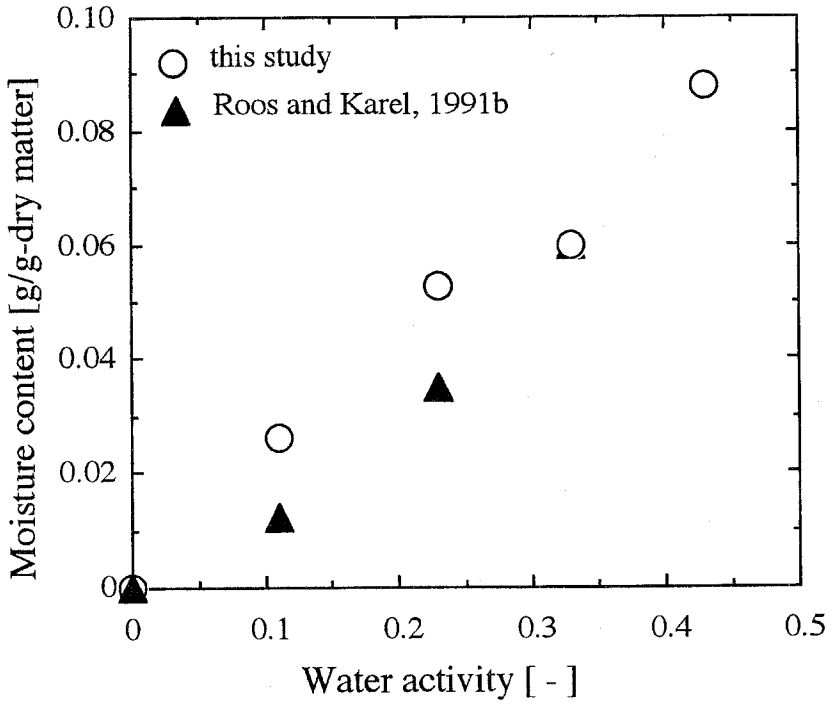


Fig. 5.1(c) Sorption isotherms of amorphous lactose

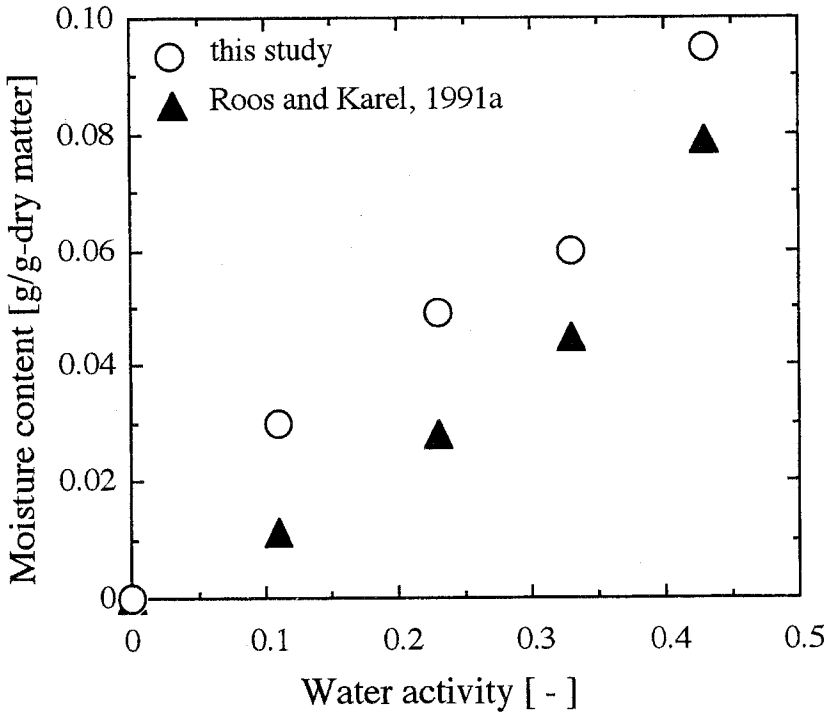


Fig. 5.1(d) Sorption isotherms of amorphous maltose

5. 3. 1. 2 Glass transition temperature of amorphous sugar samples

DSC thermograms of amorphous sugar samples are shown in Fig. 5.2. From the thermograms, T_g of amorphous sugar samples are determined and plotted against moisture contents in Fig. 5.3. In Fig. 5.3, values reported in literature (Green and Angell, 1989; Orford et al., 1990; Roos and Karel, 1990, 1991a,b,c,d; te Booy et al., 1992) are also shown. Values in this study roughly agree with those reported by Orford et al. However, values reported by Roos and Karel are lower than those in this study, and values reported by te Booy et al. are higher. This is due to differences in the definitions of T_g as shown in Fig. 5.4. The values reported by Green and Angell are lower than the values in this study. This may be due to the difference in methods of sample preparation, that is, Green and Angell adjusted moisture contents of the samples by degree of the dehydration, whereas by equilibration to saturated salt solutions in this study.

In Fig. 5.3, T_g decreases with increasing water activity. It is typical for carbohydrates (Roos and Karel, 1990, 1991a, b, c, d). Sucrose shows much lower T_g than the other three sugars. The three sugars are formed by two glucopyranoses, whereas a sucrose contains a glucofranose, that has higher conformational flexibility than glucopyranose. It is expected that molecules with higher conformational flexibility have lower T_g (Orford et al., 1990). Therefore much low T_g of amorphous sucrose is considered to be due to the higher conformational flexibility of sucrose molecule.

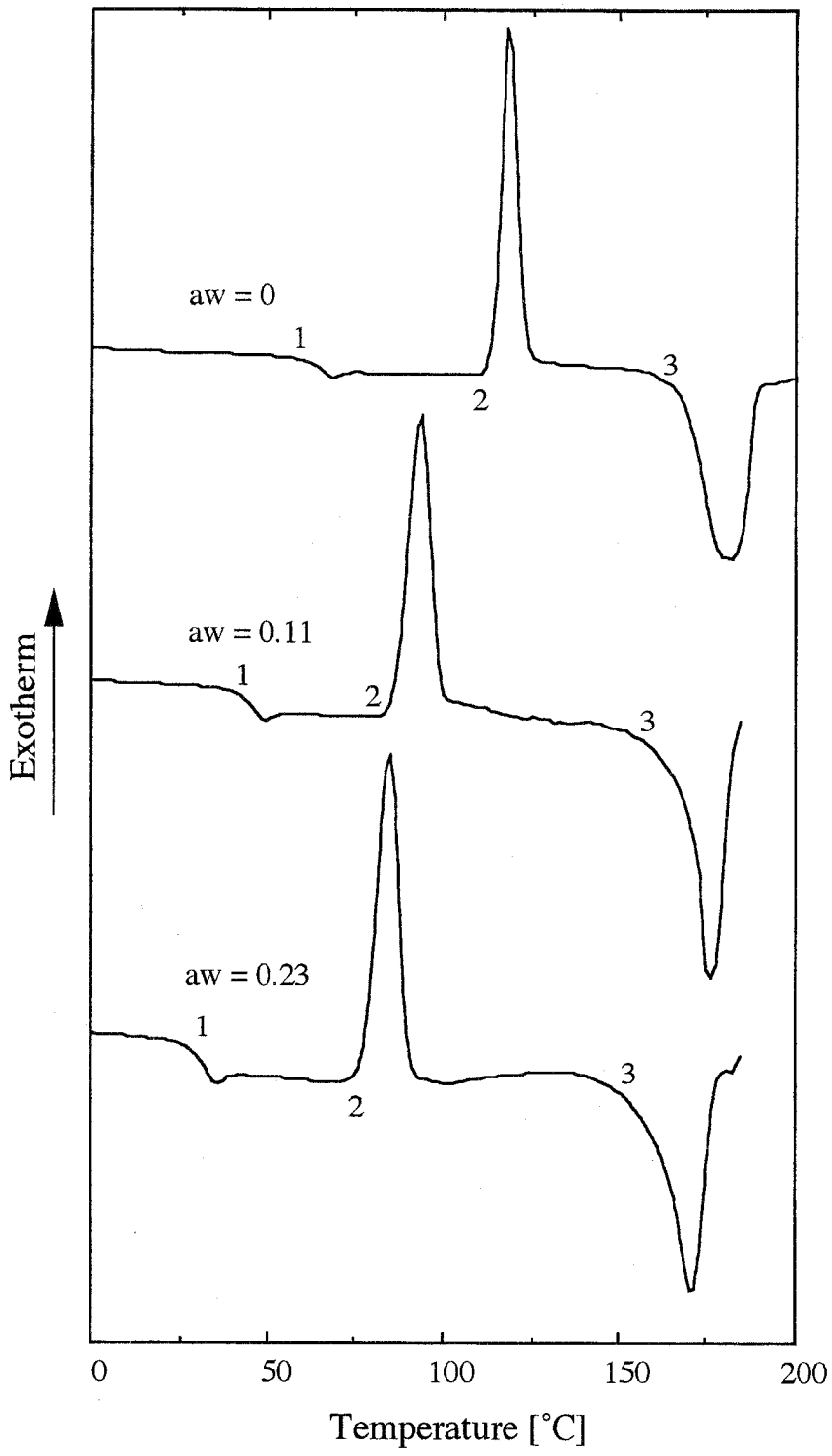


Fig. 5.2(a) DSC thermograms of amorphous sucrose equilibrated to constant water activities at 25°C. The thermograms show glass transition (1), crystallization (2), and melting (3).

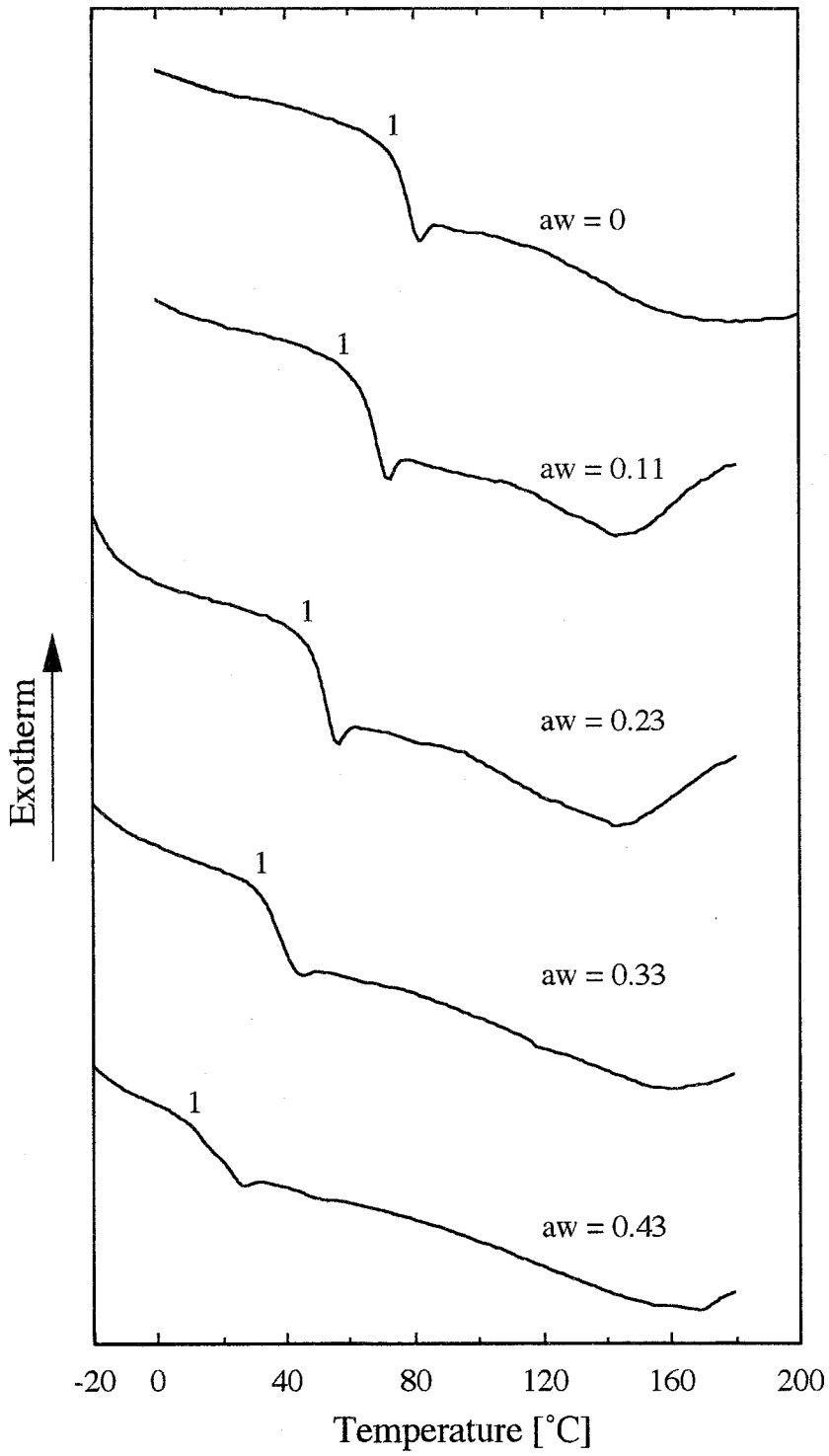


Fig. 5.2(b) DSC thermograms of amorphous trehalose equilibrated to constant water activities at 25°C. The thermograms show glass transition (1).

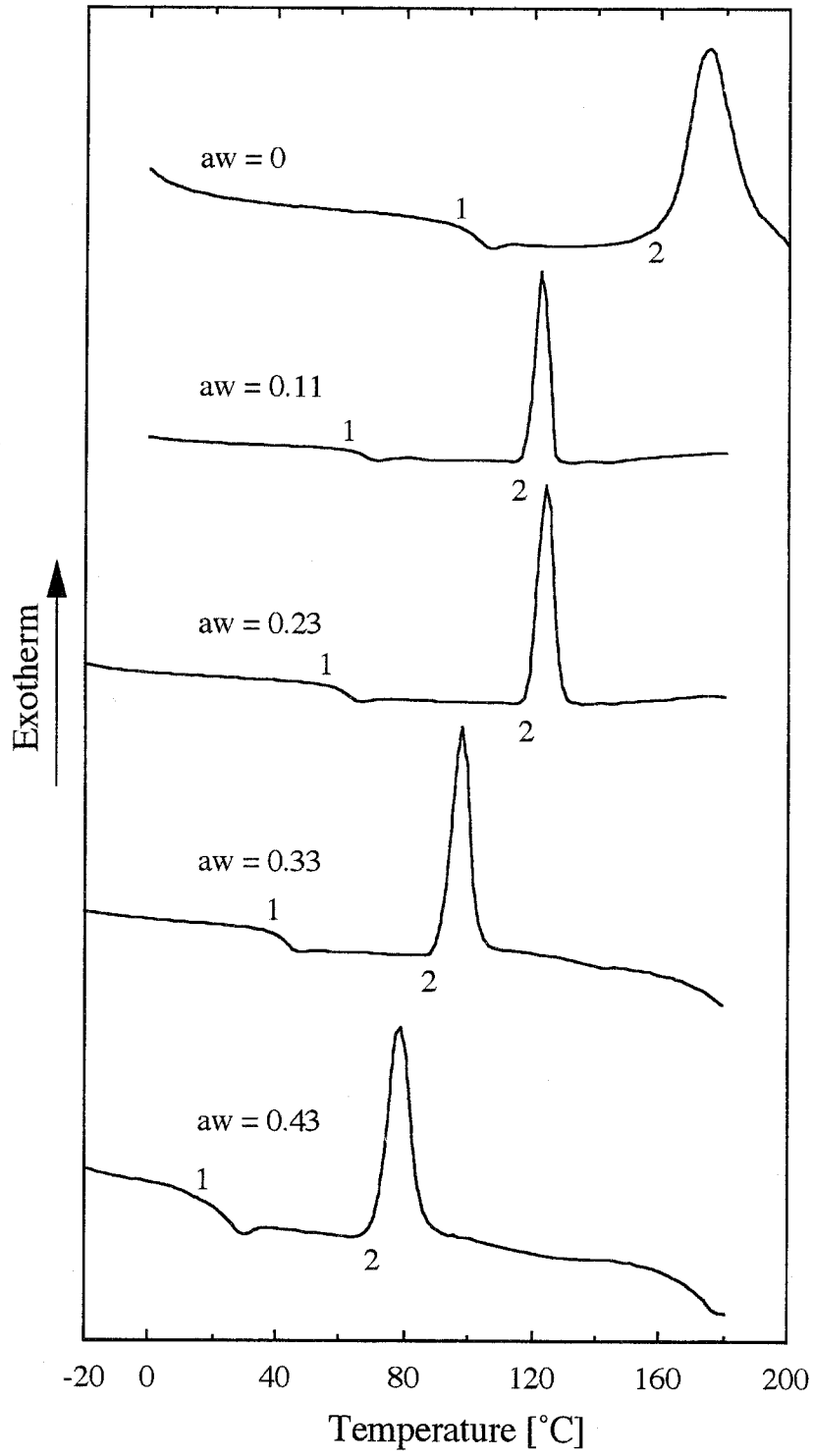


Fig. 5.2(c) DSC thermograms of amorphous lactose equilibrated to constant water activities at 25°C. The thermograms show glass transition (1) and crystallization (2).

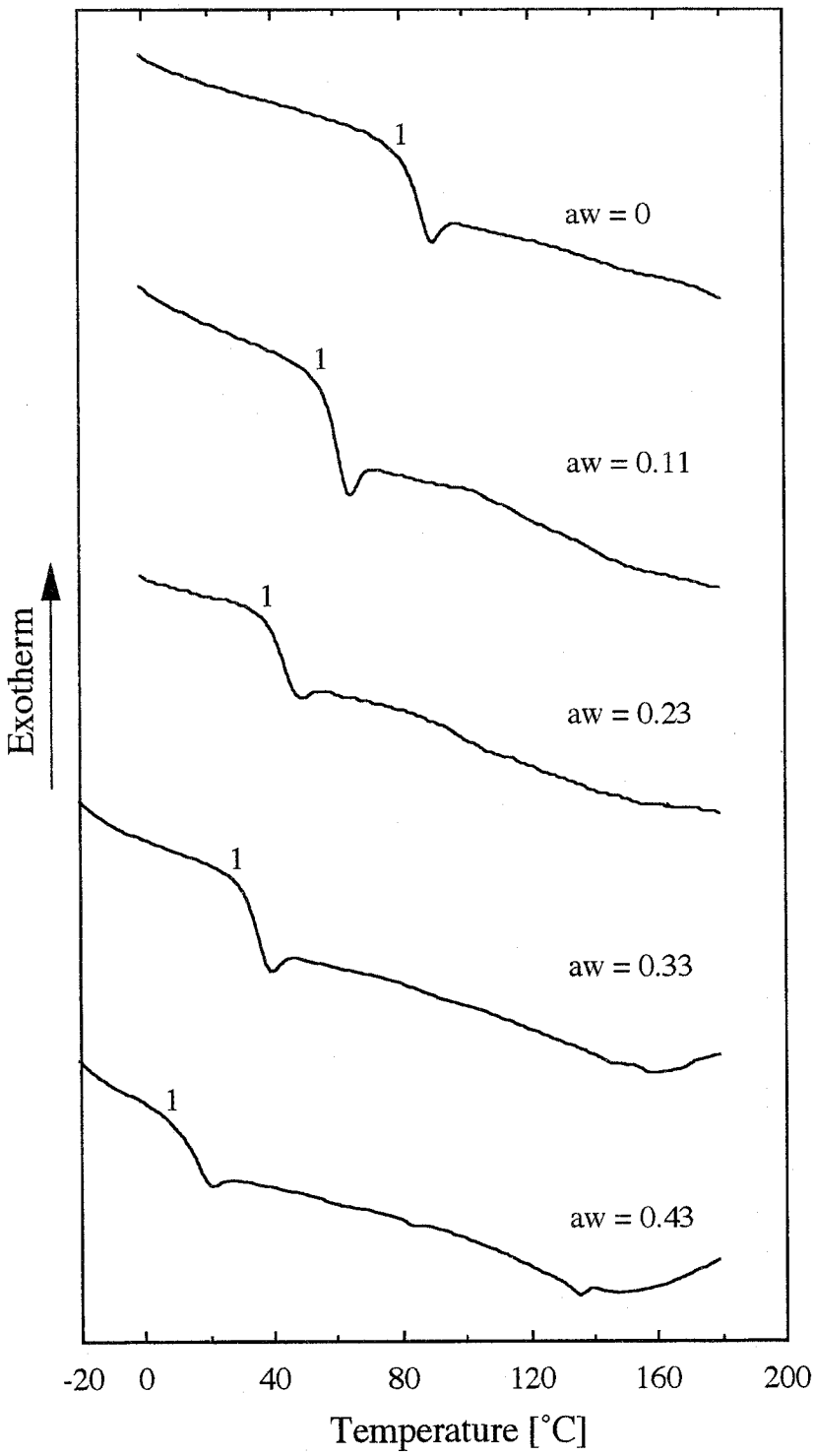


Fig. 5.2(d) DSC thermograms of amorphous maltose equilibrated to constant water activities at 25°C. The thermograms show glass transition (1).

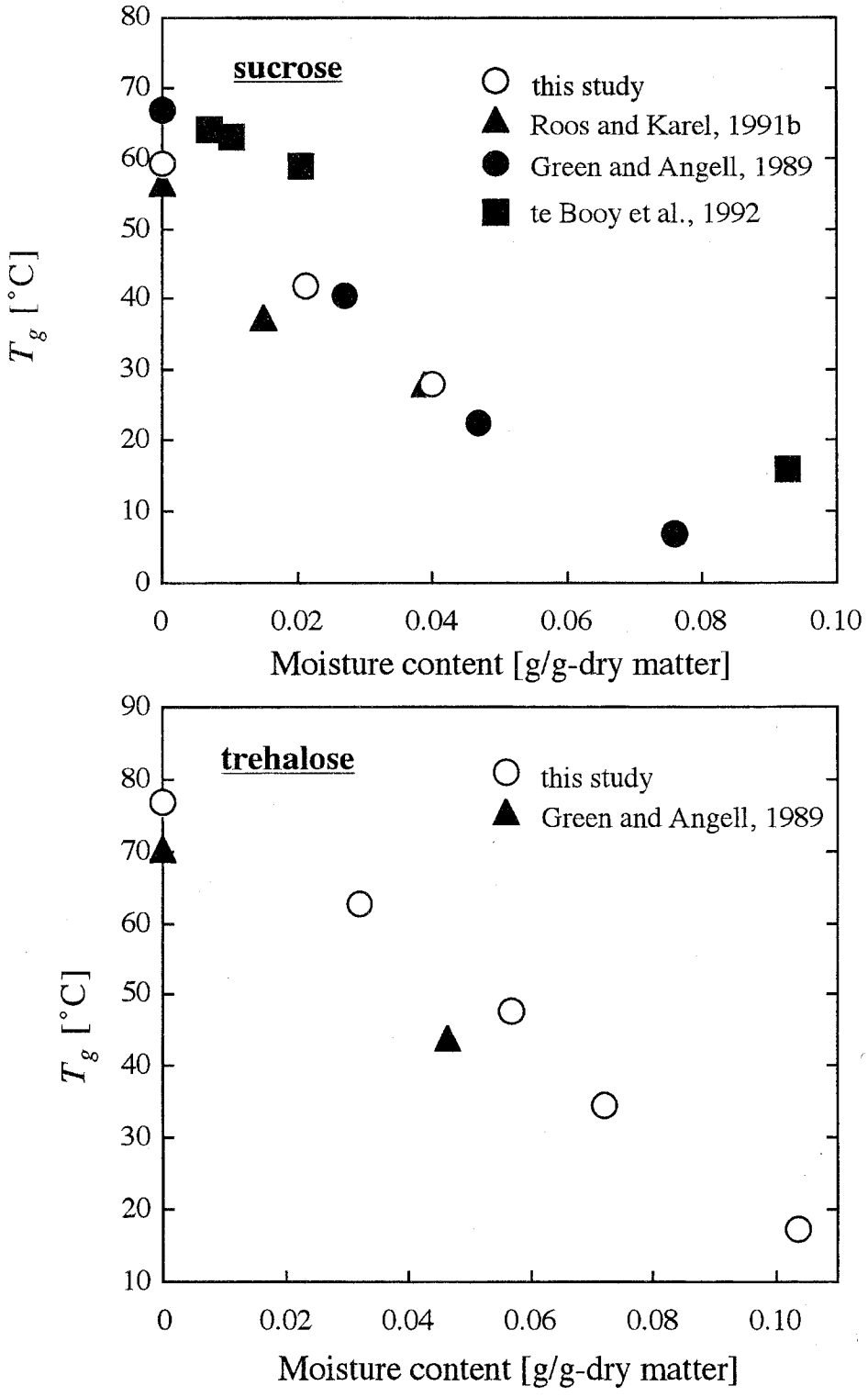


Fig. 5.3(a) Moisture content dependencies of T_g of amorphous sugars

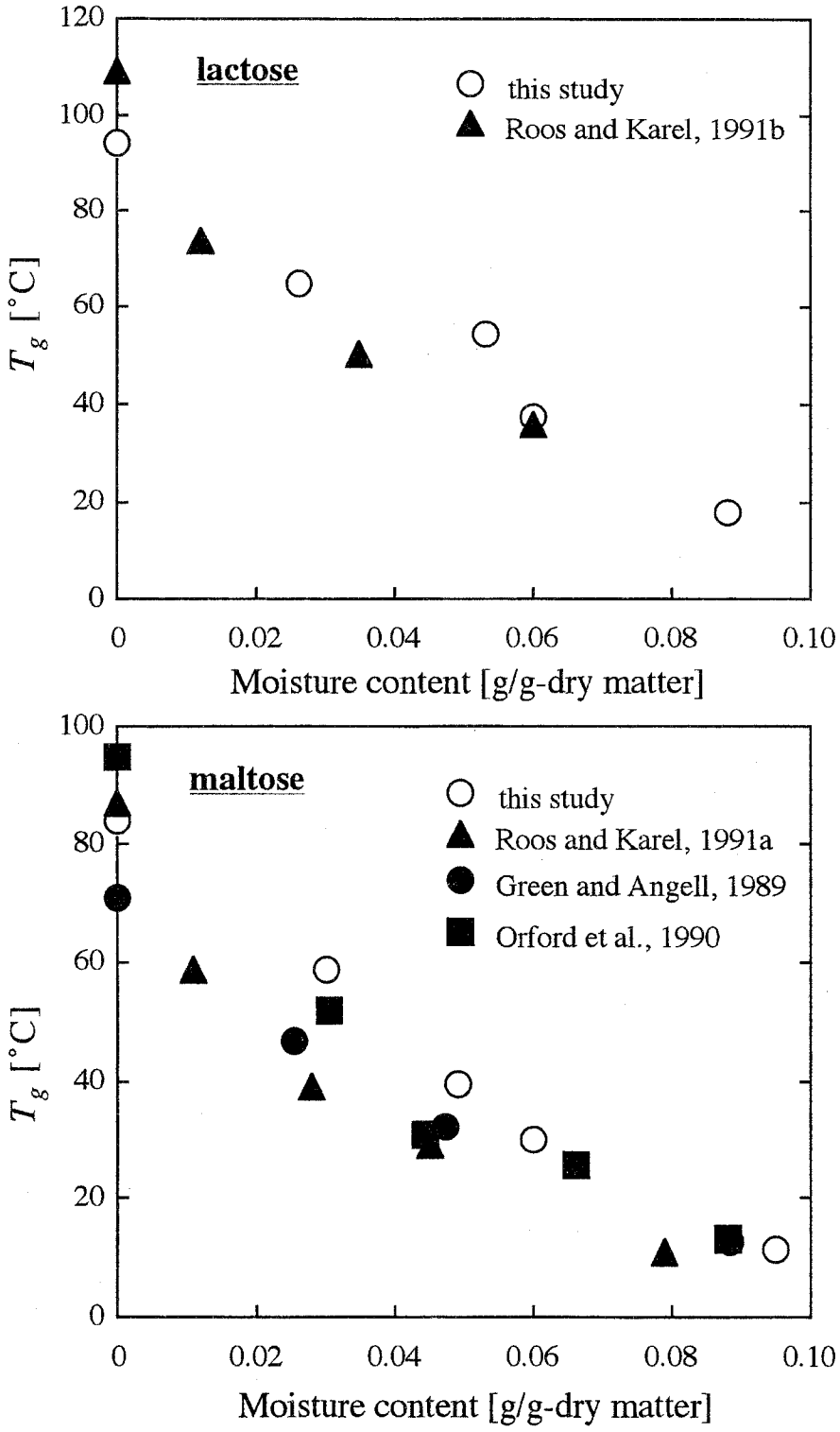


Fig. 5.3(b) Moisture content dependencies of T_g of amorphous sugars

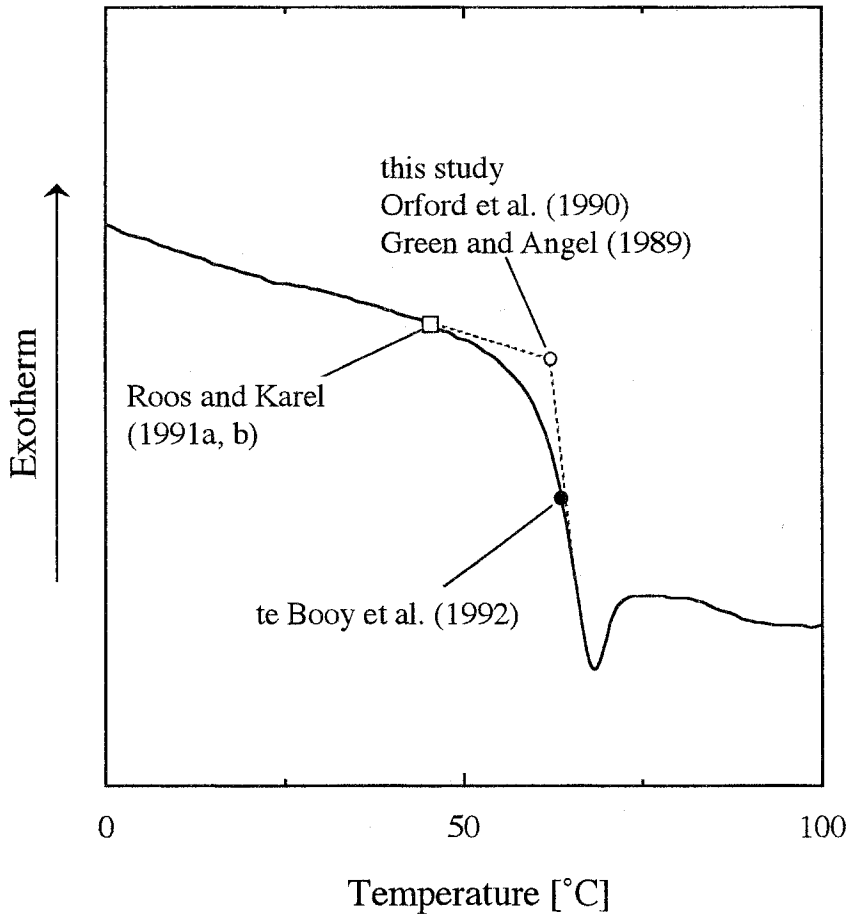


Fig. 5.4 Difference in the definition of T_g among researchers

5. 3. 1. 3 Crystallization and melting of amorphous sugar samples

According to the DSC analysis of amorphous sugar samples, amorphous sucrose showed peaks due to crystallization and melting, and amorphous lactose showed a peak due to crystallization (Fig. 5.2). Such peaks were not detected for maltose and trehalose samples. These results indicate that amorphous maltose and trehalose should be inherently hard to crystallize. Due to such a nature, trehalose and maltose may show high stabilizing effect of proteins as shown in Chapter 2.

In Fig. 5.5 T_{cr} and T_m for sucrose and T_{cr} for lactose are plotted against moisture content. In Fig. 5.6 ΔH_{cr} and ΔH_m of sucrose and ΔH_{cr} of lactose are shown. In Fig. 5.5 and 5.6, it is found that T_{cr}, ΔH_{cr}, and ΔH_m decrease with increasing the moisture content. Such tendencies are typical for carbohydrates (Roos and Karel, 1990, 1991b; te Booy, 1992). It is noticeable that T_{cr} and ΔH_{cr} of lactose bend around 0.05 g/g-lactose. Lactose is known to form two different crystalline forms, anhydrate and monohydrate (Sharp and Doob, 1942; Vuataz, 1988). Here, 0.05 g/g-lactose of water are necessary at least for the formation of monohydrate crystal. Therefore the bend of the moisture content dependency of T_{cr} and ΔH_{cr} of the amorphous lactose may be occurred because of the change of the crystalline form from anhydrate into monohydrate.

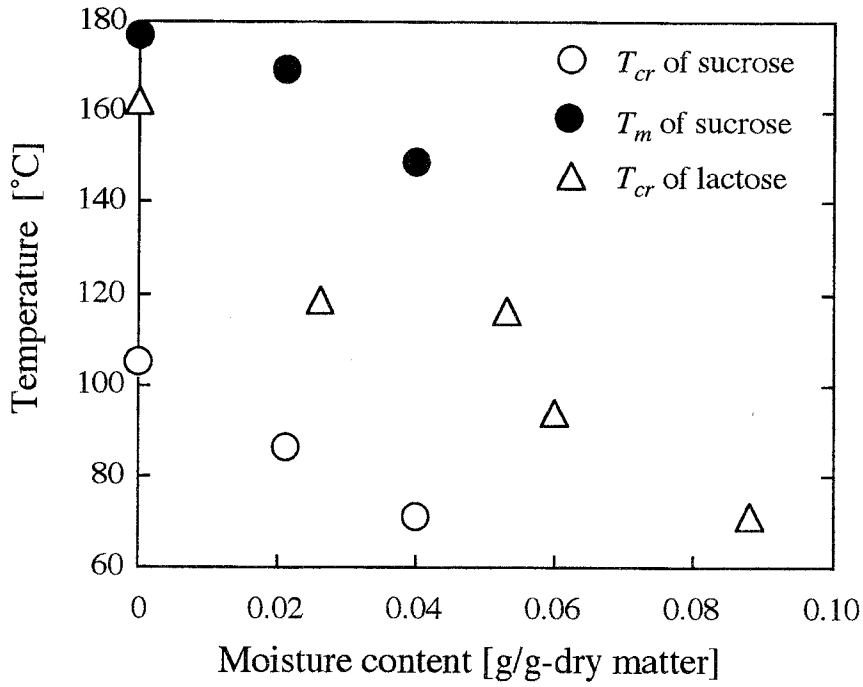


Fig. 5.5 Crystallization and melting temperature of amorphous sucrose and lactose

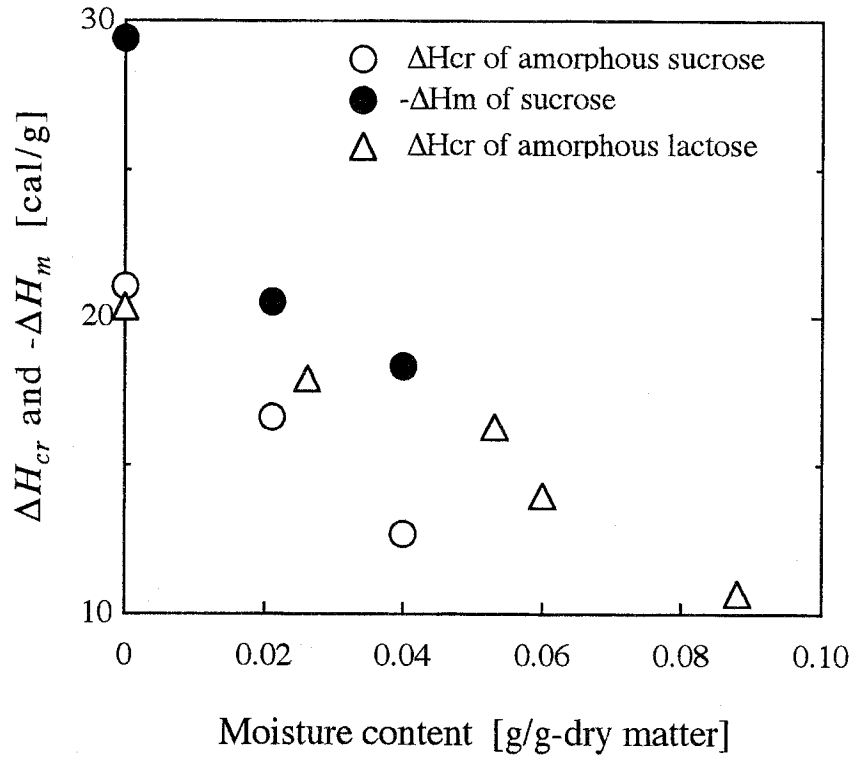


Fig. 5.6 Crystallization and melting enthalpies of amorphous sucrose and lactose

5. 3. 2 Moisture content of sugar-BSA samples

Moisture contents of sugar-BSA samples are listed in Table 5.1 as well as those of amorphous sugar samples. In most case, moisture contents of sugar-BSA samples are equal to or less than those of amorphous sugar samples, although the moisture content of BSA alone sample is higher than that of sugar alone sample. This is because the hydration level of BSA is reduced in the sugar-BSA sample by the formation of sugar-BSA hydrogen bonds, as described above chapters.

Table 5.1 Moisture contents of sugar-BSA samples equilibrated at given water activity. Values in parentheses are moisture contents of amorphous sugar samples.

water activity [-]	BSA [g/g-dry BSA]	sucrose-BSA [g/g-dry matter]	trehalose-BSA [g/g-dry matter]
0	0	0 (0)	0 (0)
0.11	0.034	0.024 (0.021)	0.033 (0.032)
0.23	0.058	0.040 (0.040)	0.058 (0.057)
0.33	0.073	0.058 (-[a])	0.067 (0.072)
0.43	0.094	0.087 (-[a])	0.082 (0.103)

water activity [-]	lactose-BSA [g/g-dry matter]	maltose-BSA [g/g-dry matter]
0	0 (0)	0 (0)
0.11	0.030 (0.026)	0.031 (0.030)
0.23	0.055 (0.053)	0.048 (0.049)
0.33	0.058 (0.060)	0.054 (0.060)
0.43	0.075 (0.088)	0.073 (0.095)

[a] not measured

5. 3. 3 DSC analysis and glass transition

DSC thermograms of sugar-BSA samples are shown in Fig. 5.7. Peaks due to crystallization and melting of sugars were not detected in all thermograms of sugar-BSA samples. This seems to be due to the hindrance of crystallization by BSA described in Chapter 4.

Thermograms of sucrose, trehalose, lactose, and maltose showed remarkable ascendance of the apparent specific heat at high temperature above 150°C. After DSC analysis, these samples were observed to turn brown and found to give off aromatic smells. From the fact, these sugars should undergo chemical reaction such as Maillard reaction (Kato et al., 1986) at high temperature during DSC analysis. In contrast, trehalose-BSA sample shows almost flat apparent specific heat even at high temperature above 150°C. This result demonstrates high chemical stability of trehalose (Roser, 1991).

In Fig. 5.8, T_g values of sugar-BSA samples obtained from the thermograms are shown with those of sugar alone samples. Concerning lactose, T_g curves of lactose-BSA sample and lactose sample cross at water activity 0.33; Comparing with T_g of lactose sample, that of lactose-BSA sample is low at lower water activity than 0.33, and high at higher water activity. Similar results are obtained for other sugars.

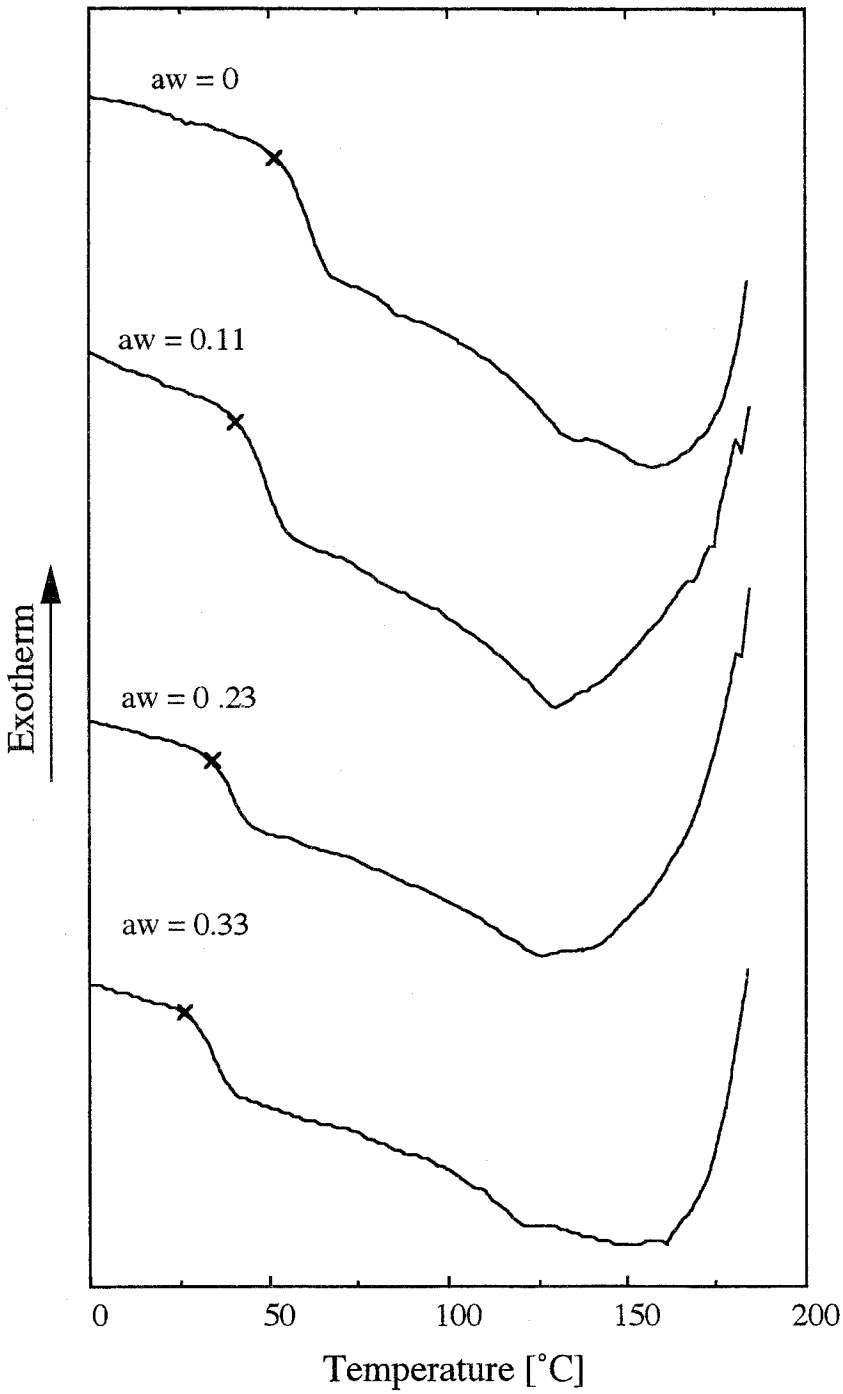


Fig. 5.7(a) DSC thermograms of amorphous sucrose containing 0.50 g/g-sugar BSA at water activity 0-0.33. The thermograms show glass transition (×).

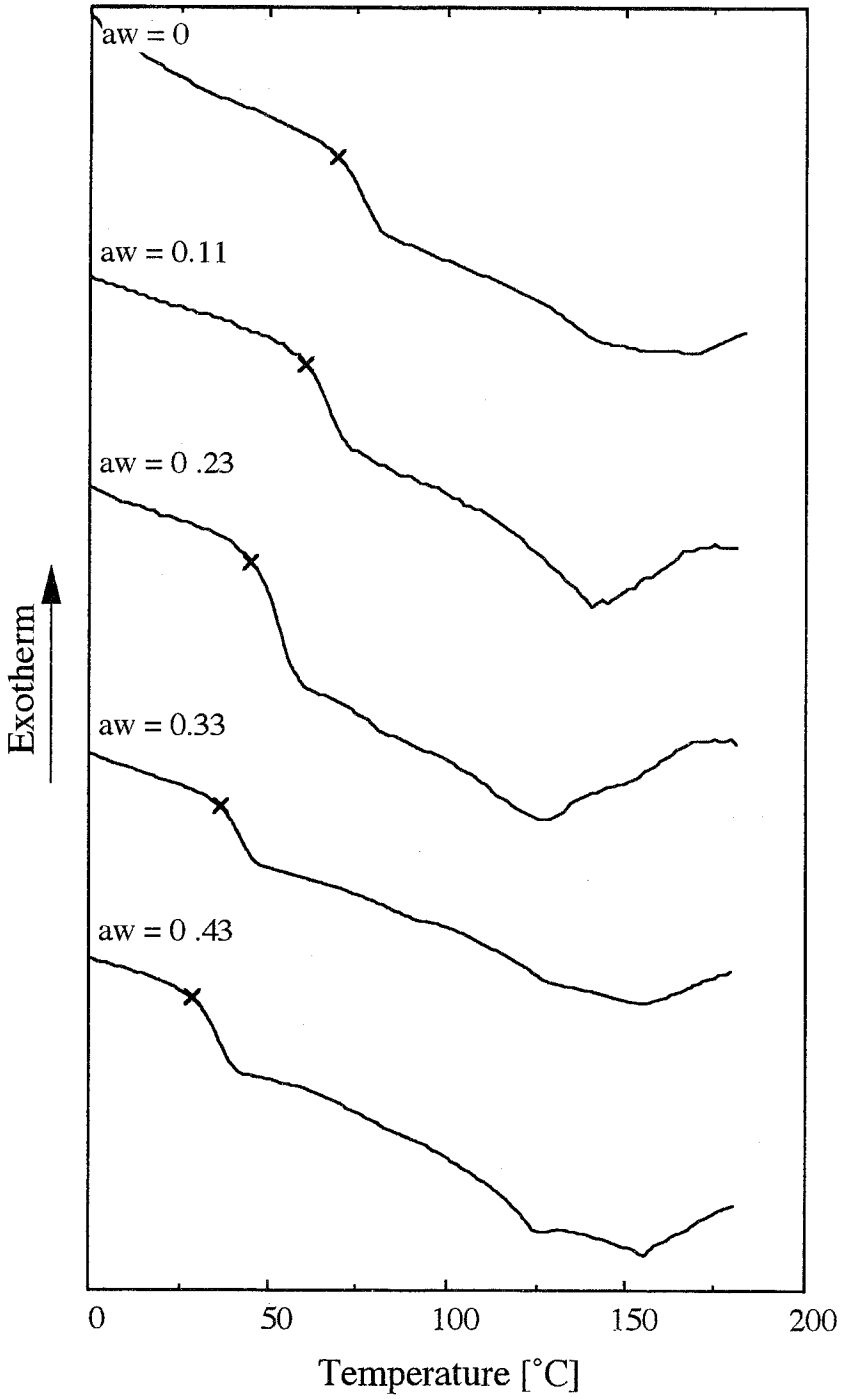


Fig. 5.7(b) DSC thermograms of amorphous trehalose containing 0.50 g/g-sugar BSA at water activity 0-0.43. The thermograms show glass transition (×).

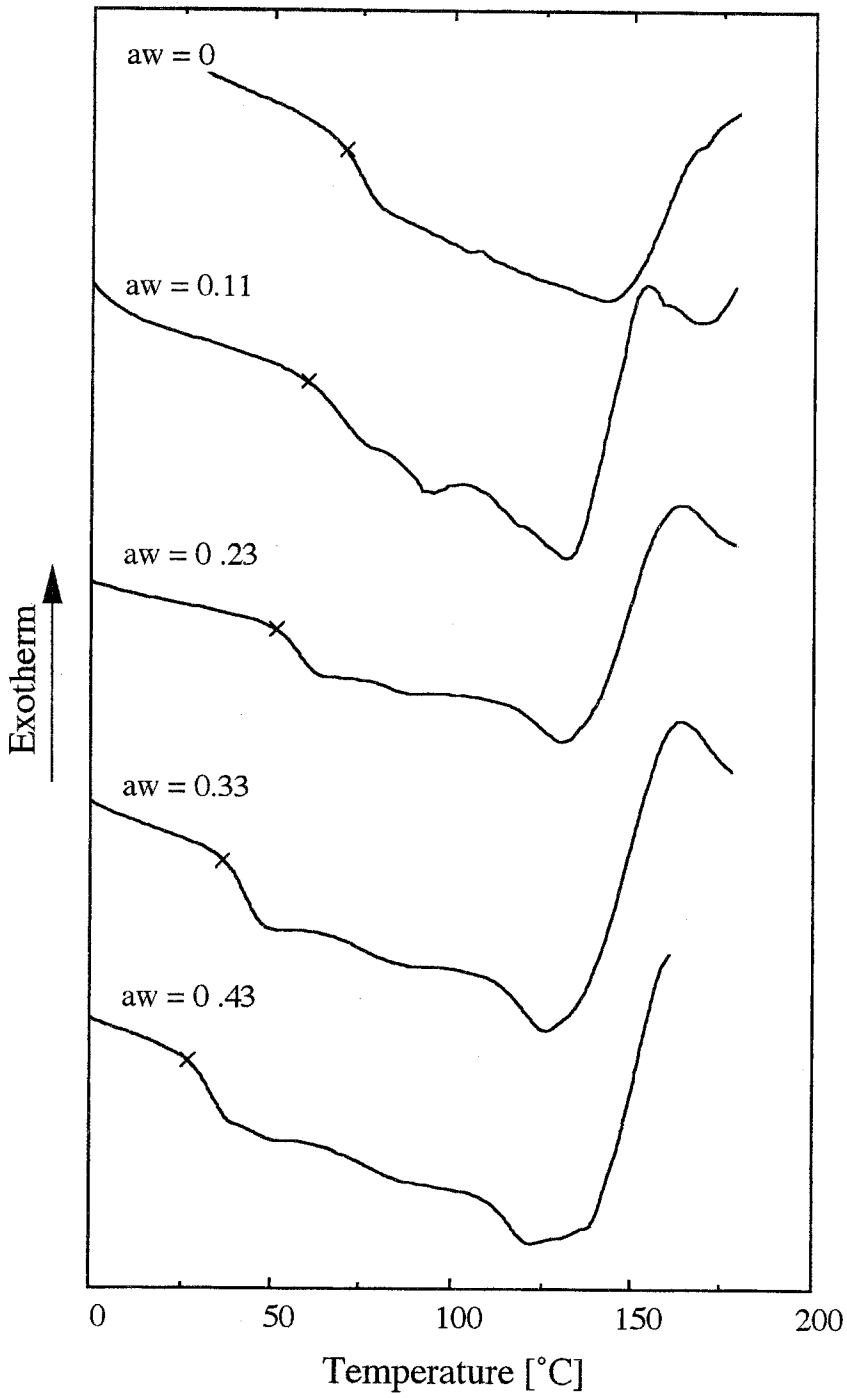


Fig. 5.7(c) DSC thermograms of amorphous lactose containing 0.50 g/g-sugar BSA at water activity 0-0.43. The thermograms show glass transition (×).

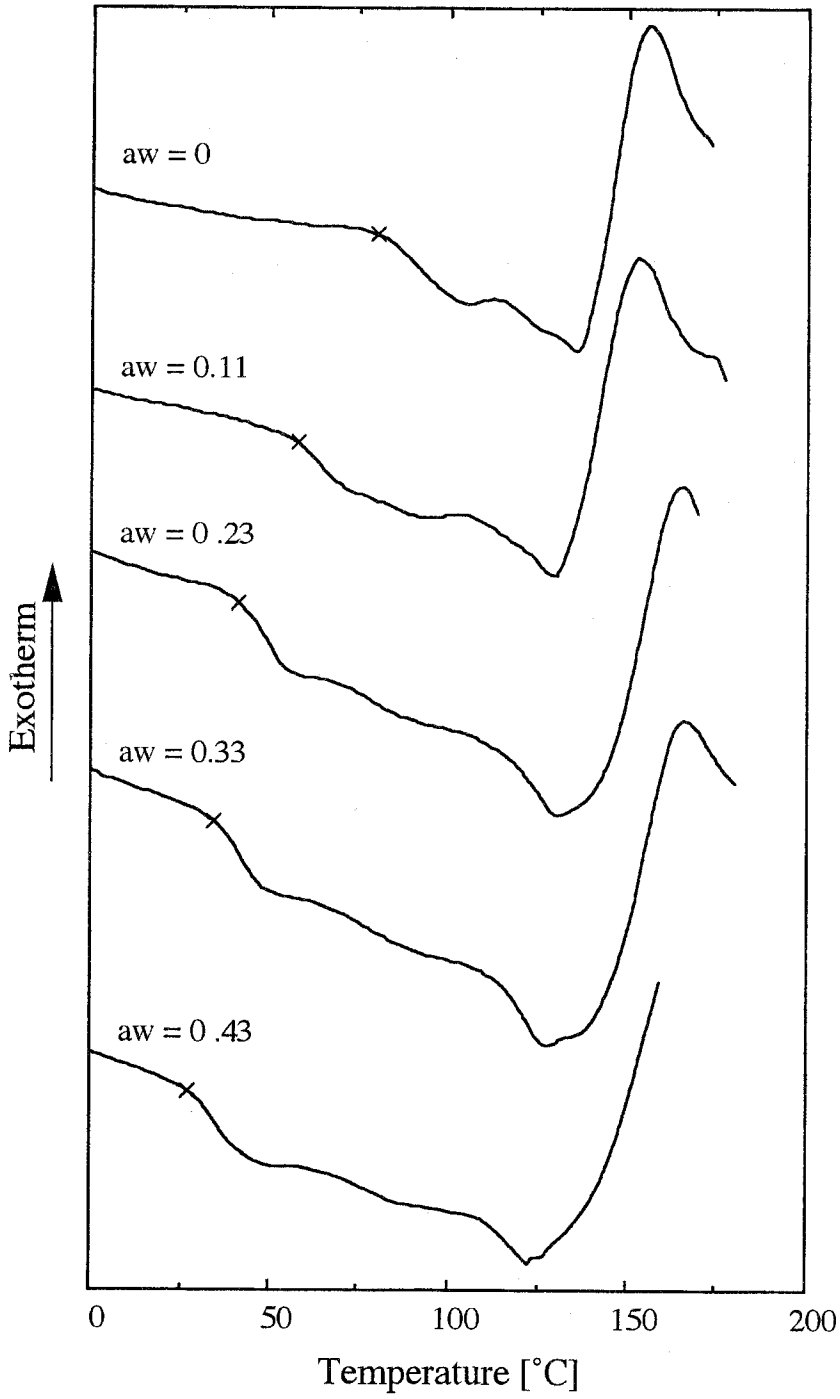


Fig. 5.7(d) DSC thermograms of amorphous maltose containing 0.50 g/g-sugar BSA at water activity 0-0.43. The thermograms show glass transition (×).

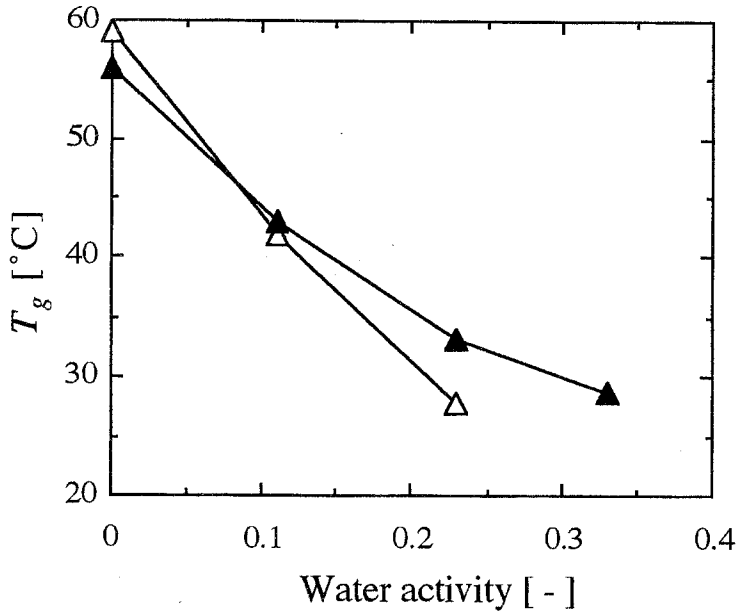


Fig. 5.8(a) Glass transition temperatures of (▲) amorphous sucrose containing 0.5 g-BSA/g-sucrose and (△) sucrose alone

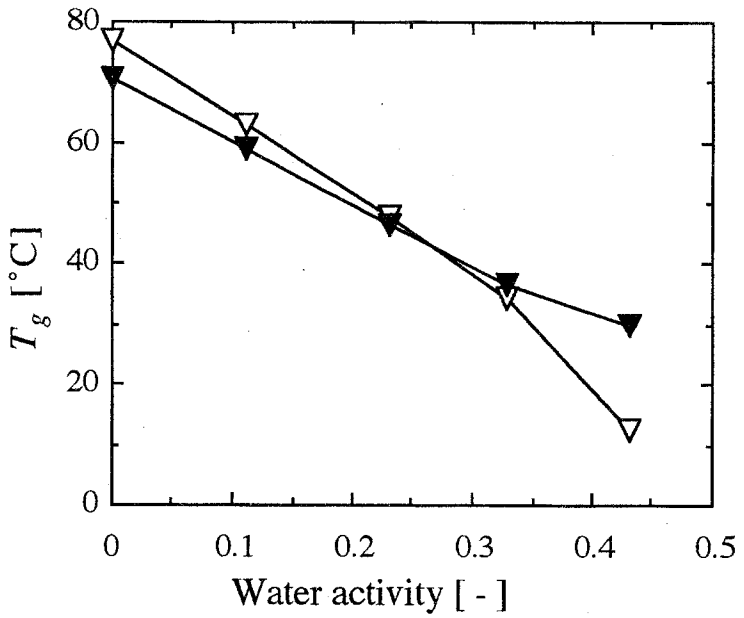


Fig. 5.8(b) Glass transition temperatures of (▼) amorphous trehalose containing 0.5 g-BSA/g-trehalose and (▽) trehalose alone

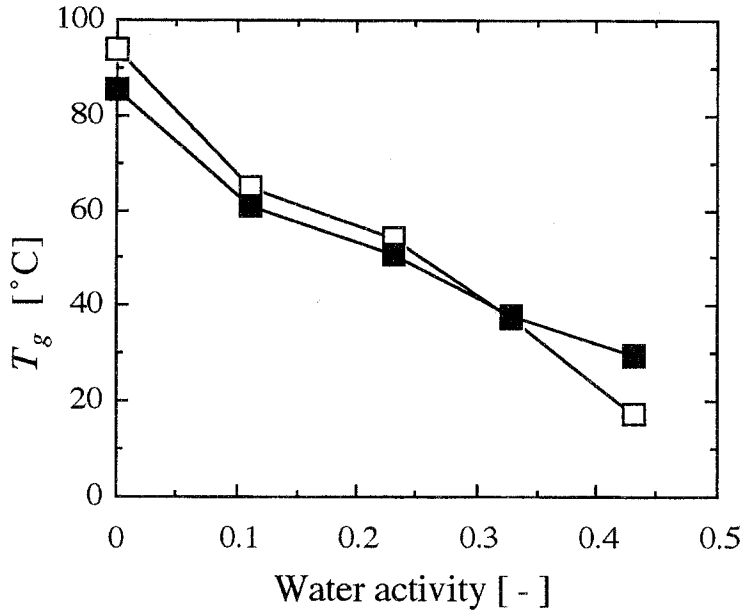


Fig. 5.8(c) Glass transition temperatures of (■) amorphous lactose containing 0.5 g-BSA/g-lactose and (□) lactose alone

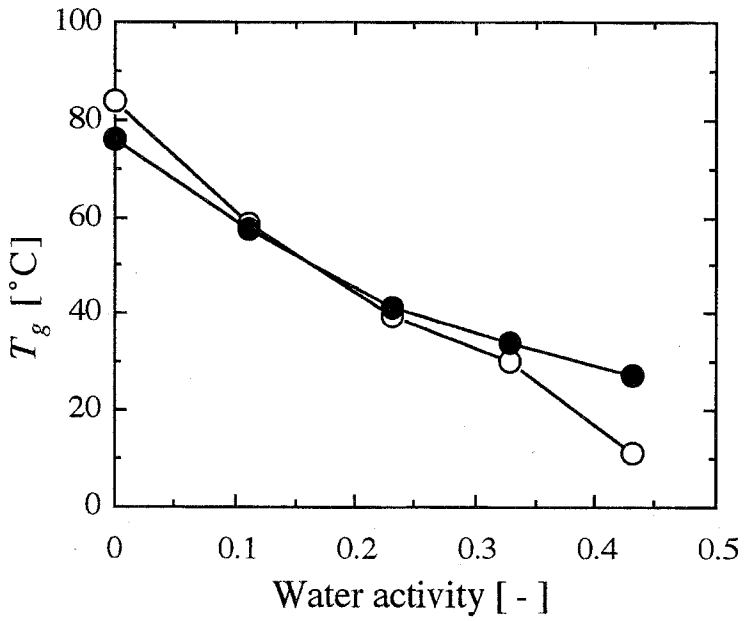


Fig. 5.8(d) Glass transition temperatures of (●) amorphous maltose containing 0.5 g-BSA/g-maltose and (○) maltose alone

Next, the author discusses the degree of the change of T_g by containing BSA. ΔT_g , the difference between T_g value of sugar alone sample (T_{g0}) and that of sugar-BSA sample, is plotted as a function of T_{g0} in Fig. 5.9. ΔT_g decreases remarkably with an increase in T_{g0}. It is noticeable that ΔT_g curves for the four sugars are almost the same. That is, ΔT_g mainly depends on T_{g0}, and does not depend on the kind of sugar so much. All the ΔT_g curves attain 0 around T_{g0} = 43°C. According to the interpretation for the effect of BSA on T_g described in the section 4.3.2.1, ΔT_g would be determined by the strength of two interacting forces, that is, intermolecular force in amorphous matrix and interacting force between BSA and amorphous matrix. Thus, these results suggest that the strength of interacting force between BSA and amorphous matrix is equivalent for the four sugars, and approximately equal to the strength of intermolecular forces for amorphous matrix whose T_g is about 43°C.

5. 4 Conclusion

The author investigated the influence of containing BSA on glass transition of amorphous sugars. T_g was lowered by containing BSA at low water activity and raised at high water activity. For glass transition, it was found that, when ΔT_g values for sugar-BSA mixtures were plotted against those for sugar alone samples (T_{g0}), ΔT_g curves for the four kinds of the mixtures were almost the same. The ΔT_g curves showed 0 when T_g of sugar alone sample was about 43°C. This result suggests that the strength of interacting force between BSA and amorphous matrix is equal to that of intermolecular forces for amorphous matrix whose T_g is about 43°C.

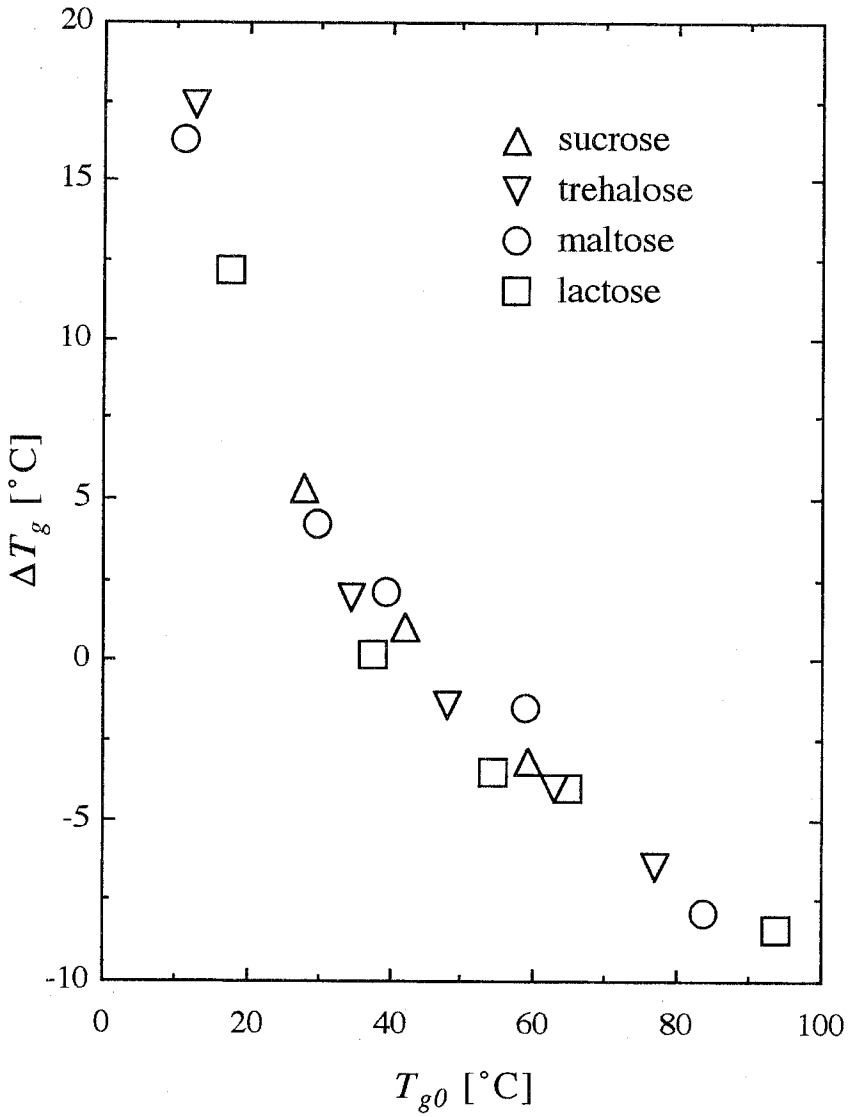


Fig. 5.9 The dependence of ΔT_g on T_{g0}. ΔT_g is the difference between the T_g value of the sample without BSA (T_{g0}) and that of the sample whose BSA content is 0.5 g/g-sugar.

Chapter 6

Conclusions and Remarks on the Future Study

6. 1 Conclusions

This thesis was dedicated to obtain basic information about the improvement of the stability of freeze-dried protein by sugars. Through the study, the following conclusions were obtained.

Chapter 2

Thermal stabilization of freeze-dried proteins by sugars was studied. Three kinds of enzymes, alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH), and malate dehydrogenase (MDH) were used as model proteins. Aqueous solutions of enzymes with sugars were freeze-dried and stored in dry air at 65°C. Stabilities of freeze-dried enzymes were remarkably improved by addition of trehalose or raffinose. By measurement of X-ray diffractometry, those sugars were found to form fully amorphous matrix in freeze-dried samples. Furthermore, sucrose stabilized enzymes highly when it was amorphous in samples, though it showed low stabilizing effect when it was crystalline. These results indicate that the stabilizing effect of sugars is closely related to the amorphous matrix formed by sugars.

Chapter 3

The role of the sugar-protein hydrogen bonds in the thermal stabilizing effect of sugar on freeze-dried protein was studied. Using sucrose and lactate dehydrogenase (LDH) as models for sugar and protein, freeze-dried LDH involved in sucrose of different degree of crystallinity were prepared. By FT-IR and XRD measurement, it was found that when sucrose was amorphous in the samples the degree of hydrogen bond formation was high and LDH was stabilized remarkably. In contrast, when sucrose was crystalline, the degree of hydrogen bond formation was low and LDH was inactivated. These results indicate that the stabilizing effect of sugar is closely related to the sugar-protein hydrogen bond. The influences of sucrose content on the thermal stabilizing effect were also investigated. It was found that there was an optimum sucrose content for the thermal stabilizing effect. This was because amorphous structure of sucrose was stabilized and prevented from crystallization by LDH. Thus we can deduce that sugars and proteins work together to maintain protein activities.

Chapter 4

In order to investigate the influence of protein on the phase transitions of sugar, the author carried out differential scanning calorimetry (DSC) analysis of freeze-dried sucrose containing various contents of water and bovine serum albumin (BSA). Glass transition temperature T_g, crystallization temperature T_{cr}, and melting temperature T_m were measured. T_g, T_{cr}, and T_m decreased with increasing water activity. Addition of BSA raised T_g except the case that water activity was low (up to about 0.1). T_{cr} increased linearly with BSA content, and T_m did not depend on BSA

content. The increase in T_g and T_{cr} with BSA suggests that protein contributes to stabilizing amorphous structure of sugar.

Chapter 5

Glass transition of four amorphous sugars (sucrose, trehalose, lactose, and maltose) containing BSA was investigated. Sugar-BSA samples were prepared by freeze-drying sugar-BSA solutions. Samples were equilibrated at several water activities ranging from 0 to 0.43. Glass transition temperature (T_g) was measured. For the all sugars, containing BSA had the same effect on glass transition: T_g was lowered at low water activity and raised at high water activity. The difference between T_g of sugar-BSA sample and that of amorphous sugar sample (T_{g0}) depended mainly on T_{g0}.

6. 2 Remarks on the Future Study

In this study, the author indicated that the hydrogen bond between sugar and protein contributes to thermal stabilization of protein in amorphous sugar. Thus the more sugar-protein hydrogen bonds are formed, the more highly protein will be stabilized. For the production of freeze-dried protein with more sugar-protein hydrogen bonds, it is indispensable to elucidate when and how sugar-protein hydrogen bonds are formed. The elucidation of the mechanism of the formation of sugar-protein hydrogen bonds will help to predict the optimum condition for production of freeze-dried protein.

The method to directly estimate the degree of formation of sugar-protein hydrogen bond is necessary to investigate the formation of sugar-protein hydrogen bond in detail. As shown in Chapter 3, from degree of the shift of

the amide bands in infrared spectra of protein, one can evaluate the degree of formation of sugar-protein hydrogen bond qualitatively. However, the quantification of formation of sugar-protein hydrogen bond seems to be impossible because the degree of shift of amide bands due to hydrogen bond is varied by the kind of protein and the molecules forming hydrogen bond with protein, sugar or water.

Nomenclature

C_A , C_B	=	weight ratio of component A and B in the mixture	[g/g-dry matter]
T_c	=	collapse temperature	[°C]
T_{cr}	=	crystallization temperature	[°C]
T_g	=	glass transition temperature	[°C]
T_{g0}	=	T_g value of sugar alone sample	[°C]
T_m	=	melting temperature	[°C]
w_A, w_B	=	moisture contents of component A and B alone	[g/g-dry matter]
W_{LDH}	=	LDH based moisture content	[g/g-LDH]
W_s	=	sugar based moisture content	[g/g-sugar]
ΔH_{cr}	=	enthalpy due to crystallization	[J/g]
ΔH_m	=	enthalpy due to melting	[J/g]
ΔT_g	=	difference between the T_g value of sugar alone and that of sugar-BSA sample	[°C]

Literature cited

Arakawa, T. and S. N. Timasheff; "Stabilization of protein structure by sugars," *Biochemistry*, **21**, 6536-6544 (1982a)

Arakawa, T. and S. N. Timasheff; "Preferential interactions of proteins with salts in concentrated solutions," *Biochemistry*, **21**, 6545-6552 (1982b)

Arakawa, T. and S. N. Timasheff; "Preferential interactions of proteins with solvent components in aqueous amino acid solutions," *Arch. Biochem. Biophys.*, **224**, 169-177 (1983)

Arakawa, T. and S. N. Timasheff; "The stabilization of proteins by osmolytes," *Biophys. J.*, **47**, 411-414 (1985)

Bakhit, R. and S. Schmidt; "Sorption behavior of mechanically mixed and freeze-dried sucrose/casein mixtures," *J. Food Sci.*, **58**, 1162-1165 (1993)

Bellows, R. J. and C. J. King; "Freeze-drying aqueous solutions: Maximum allowable operating temperature," Cryobiology, **9**, 559-561 (1972)

Brandts, J. F, *Biological Macromolecules*, vol. I (Fasman, G. D. ed), Marcel Dekker Inc., New York, N. Y., 24-72 (1968)

Careri, G., A. Giansanti, E. Gratton and Snamprogetti; "Lysozyme film hydration events: An IR and gravimetric study," *Biopolymer*, **18**, 1187-1203 (1979)

Carpenter, J. F., L. M. Crowe and J. H. Crowe; "Stabilization of phosphofructokinase with sugars during freeze-drying : Characterization of enhanced protection in the presence of divalent cations," *Biochim. Biophys. Acta.*, **923**, 109-115 (1987)

Carpenter, J. F. and J. H. Crowe; "Modes of stabilization of a protein by organic solutes during desiccation," *Cryobiology*, **25**, 459-470 (1988)

Carpenter, J. F. and J. H. Crowe; "An infared spectroscopic study of interactions of carbohydrates with dried proteins," *Biochemistry*, **28**, 3916-3922 (1989)

Carter, D. C. and J. X. Ho;"Structure serum albumin," *Adv. Protein Chem.*, **45**, 153-203 (1994)

Chinachoti, P. and M. P. Steinberg; "Interaction of sucrose with starch during dehydration as shown by water sorption," *J. Food Sci.*, **49**, 1604-1608 (1984)

Chinachoti, P. and M. P. Steinberg; "Interaction of sodium chloride with raw starch in freeze-dried mixtures as shown by water sorption," *J. Food Sci.*, **50**, 825-829 (1984)

Chinachoti, P. and M. P. Steinberg; "Interaction of sucrose with gelatin, egg albumin and gluten in freeze-dried mixtures as shown by water sorption," *J. Food Sci.*, **53**, 932-934 (1988)

Crowe, J. H., J. F. Carpenter, L. M. Crowe and T. J. Anchordoguy; "Are freezing and dehydration similar stress vectors? A comparison of modes of interaction of stabilizing solutes with biomolecules," *Cryobiology*, **27**, 219-231 (1990)

Dayhoff, M.O.; "Atlas of protein sequence and structure," National Biochemical Foundation, Washington D.C., **5**(1) (1973)

Eklund, H., Samama, J. P., Wallen, L., Branden, C. I., A. Akeson and T. A. Jones; "Structure of a triclinic ternary complex of horse liver alcohol dehydrogenase at 2.9 Å resolution," *J. Biol. Chem.*, **146,** 561-587 (1981)

Fox, T. G. and P. J. Flory; "Second-order transition temperatures and related properties of polystyrene. I. Influence of molecular weight," *J. Appl. Phys.*, **21**, 581-591 (1950)

Franks, F., R. H. M. Hately and S. F. Mathias; "Material science and the production of shelf-stable biologicals," *BioPharm*, **4**, 253-256 (1994)

Franks, F.; "Long-term stabilization of biologicals," *Biotechnology*, **12**, 38-42 (1991)

Gekko, K. and S. N. Timasheff; "Preferential hydration in glycerol-water mixtures," *Biochemistry*, **20**, 4667-4676 (1981)

Grau, U. M., W. E. Trommer and M.G. Rossmann; "Structure of the active ternary complex of pig heart lactate dehydrogenase with S-Lac-NAD at 32.7 Å resolution," *J. Mol. Biol.*, **151**, 289-307 (1981)

Green, J. L. and C. A. Angell; "Phase relations and vitrification in saccharide-water solution and the trehalose anomaly," *J. Phys. Chem*, **93**, 2880-2882 (1989)

Greenspan, L; "Humidity fixed points of binary saturated aqueous solutions," *J. Res. of NDS. A -Physics and Chem.*, **81(A)**, 89 (1977)

Hall, M. D., D. G. Levitt and L. J. Banaszak; "The crystal structure of E. Coli malate dehydrogenase: A complex of the apoenzyme and citrate at 1.87 Å resolution," *J. Mol. Biol.*, **226,** 867-882 (1992)

Hanafusa, N.; "The characteristics of protein hydration," *Japanese J. Freezing and Drying*, **33**, 62-73 (1987)

Heldman, D. R., C. W. Hall, and T. I. Hedrick; "Vapor equilibrium relationships of dry milk," *J. Dairy Sci*, **48**, 845-852 (1965)

Izutsu, K., S. Yoshioka and Y. Takeda; "The effect of additives on the stability of freeze-dried b-galactosidase stored at elevated temperature," *Int. J. Pharm.*, **71**, 137-146 (1991)

Kato, Y., T. Matsuda, N. Kato, K. Watanabe and R. Nakamura; "Browning and insolubilization of ovalubumin by Maillard reaction with some aldohexoses," *J. Agric. Food Chem.*, **34**, 351-355 (1986)

Lang, K. W. and M. P. Steinberg; "Calculation of moisture content of a formulated food system to any given water activity," *J. Food Sci.*, **45**, 1450-1452 (1980)

Lang, K. W. and M. P. Steinberg; "Linearization of the water sorption isotherm for homoheneous ingredients over aw 0.30-0.95," *J. Food Sci.*, **46**, 1450-1452 (1981)

Lee, J. C. and S. N. Timasheff; "The stabilization of proteins by sucrose," *J. Biol. Chem.*, **256**, 7193-7201 (1981)

Levine, H. and L. Slade; "Another view of trehalose for drying and stabilizing biological materials," *BioPharm*, **5(4)**, 36-40 (1992)

Manning, M. C., K. Patel and R. T. Borchardt; "Stability of protein pharmaceuticals," *Pharm. Res.*, **6**, 903-918 (1989)

Makower, B. and W. B. Dye; "Equilibrium moisture content and crystallization of amorphous sucrose and glucose," *Agr. Food Chem.*, **4**, 72-77 (1956)

Orford, P. D., R. Parker and S. G. Ring; "Aspects of the glass transition behavior of mixtures of carbohydrates of low molecular weight," *Carbohydr. Res.*, **196**, 11-18 (1990)

Ostendorp, R., G. Auerbach and R. Jaenicke; "Extrermely thermostable L(+)-lactate dehydrogenase from thermotoga maritima: Cloning, characterization, and crystallization of the recombinant enzyme in its tetrameric and octameric state," *Protein Sci.*, **5**, 862 (1996)

Poole, P. L. and J. L. Finney; "Sequential hydration of dry proteins: A direct difference IR investigation of sequence homologs lysozyme and α-lactalbumin," *Biopolymer.*, **23**, 1647-1666 (1984)

Roos, Y. and M. Karel; "DSC study of phase transitions affecting the quality of dehydrated materials," *Biotechnol. Prog.*, **6**, 159-163 (1990)

Roos, Y. and M. Karel; "Phase transitions of mixtures of amorphous polysaccharides and sugars," *Biotechnol. Prog.*, **7**, 49-53 (1991a)

Roos, Y. and M. Karel; "Plasticizing effect of water on thermal behavior and crystallization of amorphous food model," *J. Food Sci.*, **56**, 38-43 (1991b)

Roos, Y. and M. Karel; "Phase transitions of amorphous sucrose and frozen sucrose solutions," *J. Food Sci.*, **56**, 266-267 (1991c)

Roos, Y. and M. Karel; "Applying state diagrams to food processing and development," *Food Technol.*, **45(12)**, 66-71 (1991d)

Ruland, W.; "X-ray determination of crystallinity and Diffuse disorder scattering," *Acta. Cryst.*, **14**, 1180-1185 (1961)

Tant, M. R. and G. L. Wilkes; "An overview of the nonequilibrium behavior of polymer glasses," *Polym. Eng. Sci.*, **21**, 874-895 (1981)

Saltmarch, M. and T. P. Labuza; "Influence of relative humidity on the physicochemical state of lactose in spry-dried sweet whey powder," *J. Food Sci*, **45**, 1231-1243 (1980)

Slade, L. and H. Levine; "Non-equilibrium behavior of small carbohydrate-water system," *Pure & Appl. Chem*, **60**, 1841-1864 (1988)

Stevenson, C. S., A. F. Friedman, T. M. Kubiak, M. E. Donlan, and R. T. Borchardt; "Effect of secondly structure on the rate of deamidation of several growth hormone releasing factor analogs," *Int. J. Peptide Protein Res.*, **42**, 497-503 (1993)

Roos, Y; "Melting and glass transitions of low molecular weight carbohydrates," *Carbohydr,. Res*, **238**, 39-48 (1993)

te Booy, de Ruiter and de Meere; "Evaluation of the physical stability of freeze-dried sucrose-containing formulations by differential scanning calorimetry," *Pharm. Res.*, **9**, 109-114 (1992)

To, E. and M. Flink; "Collapse, a structural transition in freeze-dried carbohydrates. I. Evaluation of analytical methods," *J. Food Technol.*, **13**, 551-565 (1978)

To, E. and M. Flink; "Collapse, a structural transition in freeze-dried carbohydrates. II. Effect of solute composition," *J. Food Technol.*, **13**, 567-581 (1978)

Tsouroufis, S., J. M. Flinks and M. Karel; "Loss of structure in freeze-dried carbohydrates solutions: Effect of temperature, moisture content and composition," *J. Sci. Fd. Agr.*, **27**, 509-519 (1976)

Wang, Y. C. J. and M. A. Hanson; "Parenteral formulations of proteins and peptides: Stability and stabilizers," *J. Parent. Sci. Technol.*, **42**, 4-26 (1988)

White, G. W. and S. H. Cakebread; "The glassy state in certain sugar-containing food products," *J. Food Technol.*, **1**, 73-82 (1966)

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